

**MANUAL FOR LABORATORY DIAGNOSIS
OF COMMON EPIDEMIC PRONE DISEASES
FOR DISTRICT PUBLIC HEALTH LABORATORIES**



Integrated Disease Surveillance Project

NATIONAL CENTRE FOR DISEASE CONTROL



ABOUT THIS MANUAL

*This manual has been developed focussing primarily on outbreak prone diseases of public health importance. In addition, please refer to the previously published **'Manual of Laboratory Techniques for District Public Health Laboratories'** and **'Biosafety Manual for Public Health Laboratories'** for further information.*

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**INTEGRATED DISEASE SURVEILLANCE PROJECT
NATIONAL CENTRE FOR DISEASE CONTROL**

CONTENTS

Foreword

1. Introduction	1
2. Epidemic prone diseases under IDSP	3
- Case definitions and Laboratory Diagnosis	
3. Investigation of an Epidemic	13
4. Collection, transport and storage of specimens.....	16
5. Standard Operative Procedures.....	25
▫ <i>Cholera culture</i>	25
▫ <i>Typhoid culture</i>	30
▫ <i>TyphiDot</i>	32
▫ <i>ELISA for Dengue & Chikungunya</i>	36
▫ <i>ELISA for Hepatitis A & E</i>	38
▫ <i>Rapid test for Leptospirosis</i>	40
▫ <i>Direct detection of meningococci in CSF</i>	42
▫ <i>ELISA for Measles</i>	45
6. Antimicrobial susceptibility testing	47
7. Biosafety Measures in Laboratory	54
8. Biomedical Waste Management	64
9. Quality Assurance	68

Annexures

1. <i>Concept note – referral lab network</i>	77
2. <i>State Referral Lab plans of 9 focus states</i>	81
3. <i>List of 50 priority laboratories in 35 states under IDSP</i>	83
4. <i>Format for quarterly reporting of outbreak related tests conducted by referral labs</i>	87
5. <i>Microscopy</i>	88
6. <i>Common Culture Media</i>	93
7. <i>Common Identification Tests</i>	100
8. <i>Temperature Record of Equipment</i>	103
9. <i>References</i>	104

FOREWORD



The Integrated Disease Surveillance Project (IDSP) is a decentralized disease surveillance project, initiated by the Government of India in November 2004 with funding support from World Bank and technical support from WHO and CDC. It aims to generate and detect early warning signals of impending outbreaks and help initiate an effective response in a timely manner to minimise the impact of outbreaks.

Strengthening laboratory surveillance at the district level is an important component of this project. This document aims to provide clear and concise information on laboratory confirmation of outbreak prone diseases as envisaged under the laboratory component of IDSP. It also strives to standardise specimen collection and processing of infectious human samples for the priority diseases under the project.

The referral lab plan, currently being piloted in 9 states, aims to utilise the laboratory infrastructure and expertise available at medical colleges to diagnose and identify outbreaks in linked districts not having access to functional district public health labs. A concept note regarding the referral lab plan and final plans of the 9 states are also annexed for ready reference.

The manual, drafted by NCDC faculty and WHO experts, is based on the facilities presently available in most public health laboratories at the district level. Therefore it emphasizes the practical aspects of the diagnostic tests to be done at the district public health laboratories. Quality assurance in laboratory services to improve the reliability of lab reports has also been stressed in this manual.

Standard operative procedures (SOPs) are essential to ensure quality systems in laboratories and this manual provides a generalised SOP for the various tests under IDSP. This manual could be used as a template and modified as per local needs of the labs. Biosafety and biomedical waste management are also an important aspect of this manual and are intended to minimise healthcare associated infections, and safeguard the health of lab personnel.

I hope this manual will be useful for the laboratory personnel at the district level and help to strengthen laboratory surveillance in India.

Dr LS Chauhan
Director NCDC & PD-IDSP

Chapter 1

INTRODUCTION

Integrated Disease Surveillance Project (IDSP) was launched by Ministry of Health, Government of India in November 2004, in response to a long felt need expressed by various expert committees. IDSP is a decentralized, state based surveillance project in the country. It intends to detect early warning signals of impending outbreaks and help initiate an effective response in a timely manner.

The major components of the project are:

- 1) Integrating and decentralization of surveillance activities
- 2) Strengthening of public health laboratories
- 3) Human Resource Development – Training of State Surveillance Officers, District Surveillance Officers, Rapid Response Team, other medical & paramedical staff &
- 4) Use of Information Technology for collection, collation, compilation, analysis and dissemination of data.

IDSP is to accomplish these goals by working with the states to improve the completeness, reliability and timeliness of information collected at the peripheral levels of the health care systems.

As a part of the component of strengthening public health laboratories, initially IDSP aimed to support laboratories at 5 different levels starting from the Primary Health Centre. However, implementation experiences showed that due to limitations in availability and capacity of human resources, laboratory strengthening as envisaged was not possible. Many revisions in laboratory strengthening plan happened since then which resulted in confusion and incorrect comprehension at state levels. The laboratory cell of Central Surveillance Unit (CSU) has been pro-actively engaging the states for the final revision of the laboratory component, concentrating on basic and feasible actions.

Currently, 50 district priority laboratories in the country are being strengthened for diagnosis of epidemic prone diseases. In addition, a referral lab network is being developed in nine focus states by identifying the existing, functioning laboratories mainly in medical colleges and linking them to the adjoining districts for outbreak investigations.

Strengthening of 50 district priority labs

Fifty district priority laboratories in the country are being strengthened for diagnosis of epidemic prone diseases (the list of these 50 labs is annexed). These labs were identified by the respective state surveillance units (SSU). The labs are being

strengthened with respect to procurement of deficient equipments, hiring of a microbiologist, and provision of funds for consumables.

The labs are expected to be able to perform the tests for the investigation of the epidemic prone diseases prevalent locally. To begin with these labs are expected to perform **at least** the following tests:

- Blood culture and serological test for Typhoid (Typhi dot)
- Stool culture for cholera and other common enteropathogens
- IgM ELISA for Dengue (in endemic areas)
- ELISA / rapid test for Leptospirosis (in endemic areas)

The functioning of these labs is being monitored through the reporting of their weekly data on L forms.

Referral lab network

Under this network, services of existing functional laboratories are being used to allow access to quality laboratory services for investigation of outbreaks in the linked districts. A concept note was provided by CSU to the states for drafting the referral lab network plan. The plans were finalized through state level meetings with all the stake holders. The implementation process involves certification of labs by team of experts from the state followed by signing of MoU between SSU and referral labs and disbursement of annual grant to the referral labs. The concept note on the referral lab network and the final referral lab plans for the 9 states are annexed. The referral labs are to be monitored through quarterly reports submitted by them to the SSU and the CSU (in prescribed format is annexed).

To ensure success of the implementation, the project has provided guidelines and is focussed on obtaining laboratory test results useful for surveillance (e.g. outbreak confirmation, diagnosis of key IDSP diseases difficult to diagnose on clinical grounds), and assures continuous handholding at state and district level.

Another important aspect of IDSP is to strengthen reporting of laboratory confirmed data using L form. All the referral labs under the lab network are also to report their weekly data using the L forms. Efforts are required at the state and the district level to establish L form reporting from as many laboratories as possible in the state.

IDSP aims to strengthen the quality of lab results by integrating competency based strengthening of technical capacities of the lab personnel and quality assurance activities. Nationwide, district and state level microbiologists recruited under IDSP are being imparted training in techniques and quality assurance systems. Therefore, the Quality Assurance for public health laboratory testing is being supported by developing standard operating procedures, the external quality assessment system, biomedical waste management, development of guidelines for quality of kits as well as sample collection, transportation and handling.

Chapter 2

EPIDEMIC PRONE DISEASES UNDER IDSP

CASE DEFINITIONS & LABORATORY DIAGNOSIS

CHOLERA

Cholera is an acute diarrhoeal infection caused by ingestion of the bacterium *Vibrio cholerae*. Transmission occurs through direct faecal-oral contamination or through ingestion of contaminated water or food. The disease is characterized in its most severe form by a sudden onset of acute watery diarrhoea that can lead to death by severe dehydration and kidney failure. The extremely short incubation period - two hours to five days - enhances the potentially explosive pattern of outbreaks, as the number of cases can rise very quickly. About 75% of people infected with cholera do not develop any symptoms. However, the cholera vibrios persist in their faeces for 7 to 14 days and are shed back into the environment, potentially infecting other individuals. Cholera is an extremely virulent disease that affects both children and adults. Unlike other diarrhoeal diseases, it can kill healthy adults within hours. Individuals with lower immunity, such as malnourished children or people living with HIV, are at greater risk of death if infected by cholera.

Clinical case description

A patient with acute watery diarrhoea and severe dehydration (lethargy, altered consciousness and decreased urine output) with or without vomiting.

Case classification

Probable:

- *In an area where the disease is not known to be present:* Severe dehydration or death from acute watery diarrhoea in a patient aged 5 years or more
- *In an area where Cholera is endemic:* Acute watery diarrhoea, with or without vomiting in a patient aged 5 years or more.
- *In an area where there is a Cholera epidemic:* Acute watery diarrhoea, with or without vomiting, in any patient.

Confirmed: A probable case that is laboratory confirmed

- *Laboratory criteria for diagnosis:* Isolation of *Vibrio cholerae* 01 or 0139 from the stools samples of any patient with diarrhoea.

Key issues

1. Treatment of cholera does not depend on the results of laboratory examination. However, laboratory examination of specimens from the first few suspected

cases is important to confirm the diagnosis and to determine the characteristics of the organism.

2. A sufficient number of stool specimens should be examined to identify the causative organism. Once the presence of cholera is confirmed, *it is not necessary to examine specimens from all cases or contacts*. In fact, this should be discouraged since it unnecessarily burdens the laboratory and is not required for effective treatment.
3. Specimens should be collected before the patient has received any antibiotics.
4. Full particulars of the patient(s) from whom samples have been collected must be sent along with the samples as many factors can influence the results of the laboratory tests. The information that should accompany each stool sample is given below:
 - Name, age, sex
 - Name of mother or father
 - Address
 - Date of onset of symptoms
 - Provisional diagnosis
 - Clinical outcome (recovered, under treatment, dead, not known)
 - Antibiotic received prior to collection of sample - Y/N/not known
 - Date of sample collection

Notification of cholera cases

1. Cholera is endemic in India and several outbreaks of the disease have been reported. Because cholera has the potential of rapid spread leading to an acute public health problem, special attention is required to be given to the surveillance and prompt follow up action on reported cases of cholera.
2. A suspect case of cholera must be notified immediately by messenger, telephone or fax to the local health office. Weekly notification of confirmed cholera cases is required to be made by the state health authorities to the Directorate General of Health Services:
 - *Director, Central Bureau of Health Intelligence,*
Pushp Bhavan, Madangir Road, New Delhi – 110062A copy should be endorsed to
 - *Director, National Centre for Disease Control*
(formerly known as National Institute of Communicable Diseases),
22 Shamnath Marg, Delhi – 110054
Phone: 011-23971272, 23971060, 23913148
Fax: 23922677
3. If appropriate measures are taken, cholera remains restricted to a limited habitation. Therefore, reporting of taluka and district help in identifying the affected area.
4. The first suspect case of cholera in the area must be notified immediately to the local health officer. Laboratory confirmation should be obtained at the earliest opportunity and the results intimated to local health office as soon as these become available.

ENTERIC FEVER

Enteric / typhoid fever is a septicaemia caused by *Salmonella sp.* In India, it is caused mainly by *Salmonella typhi* and less frequently by *Salmonella paratyphi A*. It manifests in the form of fever (step-ladder rise) accompanied with other symptoms like abdominal pain, vomiting, headache, loss of appetite, etc.

Salmonella are gram negative bacteria, non acid-fast, non-capsulated and non-spore forming motile bacilli, that grow rapidly on ordinary media and optimum temperature of growth is 37°C.

Clinical case description

Insidious onset of continuous fever, headache, malaise and loss of appetite usually with gastrointestinal symptoms of more than one week duration having two or more of the following:

- Toxic look
- Coated tongue
- Relative bradycardia
- Splenomegaly
- Exposure to a confirmed case
- Clinical presentation with complications e.g. GI bleeding, perforation, etc

Case definitions

Probable case: A case compatible with the clinical description

Confirmed case: A probable case that is laboratory confirmed by:

- Isolation of *Salmonella typhi* / *paratyphi A* from the blood or any other clinical specimen
- Serology
 - Demonstration of four-fold rise in anti-salmonella antibodies in the patient's serum
 - Detection of specific IgM antibodies

DENGUE FEVER / DENGUE HAEMORRHAGIC FEVER

Dengue infection is caused by one of the four dengue virus serotypes – dengue 1, 2, 3 or 4. Dengue virus belongs to the family Flaviviridae. Annually, dengue virus infections are responsible for up to 100 million cases of dengue fever and more than 500,000 cases of dengue hemorrhagic fever.

Dengue viruses are transmitted to humans through the bite of infected *Aedes* mosquito. Once infected the mosquito remains infected for life, transmitting the virus to susceptible individuals during feeding.

Clinical case description of Dengue fever

An acute febrile illness of 2-7 days duration with 2 or more of the following:

- Headache
- Retro-orbital pain
- Myalgia
- Arthralgia
- Rash
- Haemorrhagic manifestations
- Leucopaenia



A female Aedes aegypti mosquito engorged with blood while feeding

Probable case - A case compatible with clinical description and with one or more of the following:

- Supportive serology (comparable IgG EIA titre or positive IgM antibody test on a single acute (preferably after 5 days) or convalescent-phase serum specimen)
- Epidemiologically linked with a confirmed case of dengue fever (occurrence at the same location and time as other confirmed cases of dengue fever)

Confirmed case

- Isolation of dengue virus from blood or tissue samples
- Demonstration of a four-fold rise in dengue specific antibody titres in paired serum samples
- Demonstration of dengue virus antigen in serum or tissue samples
- Detection of viral RNA in blood or tissue samples by polymerase chain reaction (PCR)

Case definition of DHF

A probable or confirmed case of dengue with the following signs:

- Haemorrhagic tendencies evidenced by one or more of the following:
 - Positive tourniquet test
 - Petechiae, ecchymoses or purpura
 - Bleeding from mucosa, gastrointestinal tract, injection sites etc
 - Haematemesis or melaena
- **And** thrombocytopenia (100,000 platelets or less per mm³)



A typical positive result from a tourniquet test (>20 petechiae per square inch)

- *And* evidence of plasma leakage due to increased vascular permeability, manifested by one or more of the following:
 - 20% rise in average haematocrit for age and sex
 - 20% drop in haematocrit following volume replacement treatment compared to baseline
 - Signs of plasma leakage (pleural effusion, ascites, and hypoproteinaemia)

ACUTE VIRAL HEPATITIS

Acute viral hepatitis is an inflammatory disease of the liver due to a viral infection and the most common cause is infection with one of 5 viruses, called hepatitis A, B, C, D, and E. Of these, only hepatitis A virus (HAV) and hepatitis E virus (HEV) are enterically transmitted. They cause acute and generally self limiting infections without any long term carrier state. However, they cause significant morbidity and socio-economic loss in many parts of the world.

Hepatitis A is caused by infection with the hepatitis A virus (HAV), a non-enveloped, positive stranded RNA virus, first identified by electron microscopy in 1973, classified within the genus hepatovirus of the picornavirus family.

Hepatitis E is caused by infection with the hepatitis E virus (HEV), a non-enveloped, positive-sense, single stranded RNA virus, type species of the Hepevirus, genus within the family Hepeviridae.

Mode of transmission

Both HAV & HEV are transmitted from person-to-person via the faeco-oral route.

HAV is abundantly excreted in faeces and can survive in the environment for prolonged periods of time. It is acquired by ingestion of faeces-contaminated food or water. Direct person-to-person spread is common in poor hygienic conditions. Occasionally, HAV is also acquired through sexual contact (anal-oral) and blood transfusions.

Hepatitis E is a waterborne disease, and contaminated water or food supplies are commonly implicated in major outbreaks.

Clinical case description

Acute illness compatible with following clinical description

- Jaundice
- Dark urine
- Anorexia, malaise
- Extreme fatigue and
- Right upper abdominal quadrant pain.

Biological signs include increased urine urobilinogen and >2.5 times the upper limit of serum alanine aminotransferase

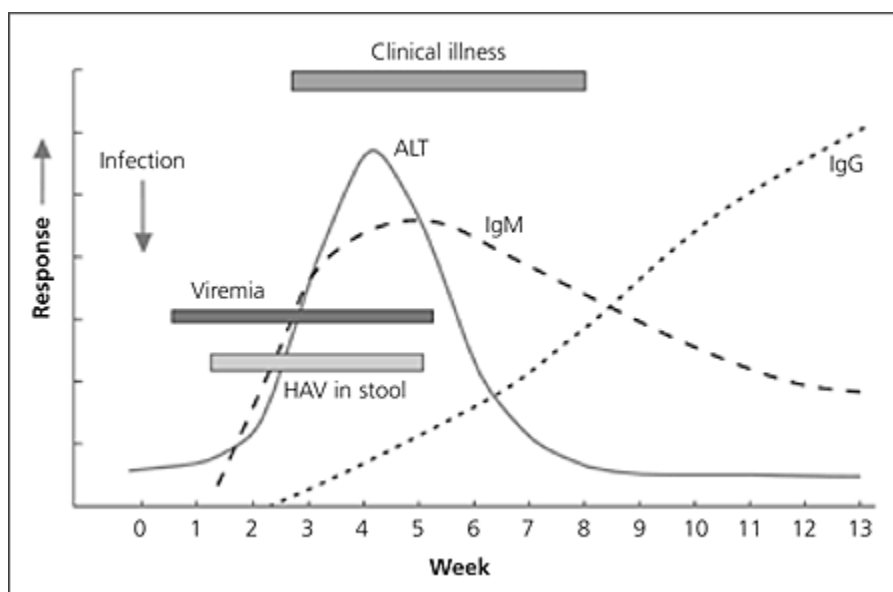
Case classification

Probable: As per clinical case definition

Confirmed: A probable case that is laboratory-confirmed

- Hepatitis A : IgM anti-HAV positive
- Hepatitis E : IgM anti-HEV positive

The mainstay of lab diagnosis rests on detecting IgM antibodies (against Hepatitis A or Hepatitis E virus) in blood of patients after 7-10 days of the infection.



*Timing of sample is important when performing serologic testing;
IgM antibodies begin to rise about a week after infection*

LEPTOSPIROSIS

During the past decade many zoonotic diseases have emerged and resulted in epidemics causing significant morbidity and mortality in human beings in different parts of India's coastal belt due to the rapid ecological changes. Outbreaks of leptospirosis have been reported from coastal districts of Gujarat, Maharashtra, Kerala, Tamil Nadu and Andaman & Nicobar Islands. Cases have also been reported from Goa, Karnataka, Orissa and Andhra Pradesh. States and union territories like West Bengal, Lakshwadeep, etc from where the disease has not been reported, may be affected in future. The deltas of Godavari, Krishna, Mahanadi and Cauveri in addition to Ganga may also be future potential areas for Leptospirosis.

Infection is acquired through contact with the environment contaminated with urine of an animal that is a carrier or is suffering from the disease caused by leptospire.

Infection may also arise from bathing or accidental immersion in the fresh water lakes, or rivers, contaminated with the urine of the infected livestock that has been using the water for drinking or wallowing. Water may also get contaminated with rodent nests located on the banks especially after heavy rainfalls and inundations.

Clinical case description

Acute febrile illness with headache, myalgia and prostration associated with any of the following symptoms:

- Conjunctival suffusion
- Meningeal irritation
- Anuria or oliguria and / or proteinuria
- Jaundice
- Haemorrhages (from the intestine / lung)
- Cardiac arrhythmia or failure
- Skin rash

AND

- History of exposure to infected animals or an environment contaminated with animal urine

Other common symptoms include nausea, vomiting, abdominal pain, diarrhoea, and joint pains.

Case classification

Probable: A case that is compatible with the clinical description

Confirmed: A probable case that is laboratory confirmed.

Laboratory diagnosis depends on:

1. The isolation of leptospira from clinical specimens confirms the clinical diagnosis. Isolation is recommended from blood or CSF during first week of illness and from urine after 10 days. Leptospire can be isolated from:
 - Blood
 - Urine
 - CSF
 - Aqueous humour
 - Amniotic or peritoneal fluids or
 - Autopsy tissues such as kidney or liver
2. Serological diagnosis
 - Leptospira agglutination test, preferably Microscopic Agglutination Test (MAT)
 - IgM ELISA / other IgM antibody tests

MEASLES

Measles is a highly contagious disease caused by the measles virus, which is a paramyxovirus belonging to the genus Morbillivirus. It is a leading cause of death

among young children globally, despite the availability of a safe and effective vaccine.

Measles virus spreads by coughing and sneezing, close personal contact or direct contact with nasal or throat secretions from an infected person. It can also spread by airborne aerosolised droplet nuclei or by contact with freshly contaminated articles. It is a highly communicable disease, with a secondary attack rate of 90% among susceptible persons. It can be transmitted by an infected individual from four days prior to the onset of the rash to four days after the rash erupts.

Clinical case description

Any person with:

- Fever, and
- Maculo-papular (i.e. non-vesicular) rash lasting for more than 3 days and
- Cough or coryza (i.e. running nose) or conjunctivitis (i.e. red eyes)



Measles rash

Case classification

- **Probable:** A case that meets the clinical case description
- **Confirmed:** a case compatible with clinical description and is laboratory confirmed or linked epidemiologically to a lab confirmed case

Laboratory diagnosis depends on

- Isolation of measles virus **OR**
- A four-fold rise in serum antibody titre **OR**
- Presence of measles-specific IgM antibodies (without a history of recent vaccination)

MENINGOCOCCAL MENINGITIS

Meningitis is an infection of the meninges, the thin lining that surrounds the brain and the spinal cord. Several different bacteria can cause meningitis and *Neisseria meningitidis* is one of the most important because of its potential to cause epidemics.

Meningococcal disease was first described in 1805 when an outbreak swept through Geneva, Switzerland. The causative agent, *Neisseria meningitidis* (also known as meningococcus), was identified in 1887. Thirteen subtypes or serogroups of *N. meningitidis* have been identified and four (*N. meningitidis* A, B, C and W135) are recognized to cause epidemics. Likewise in our country, the commonest serogroup involved in meningococcal meningitis is 'A' followed by 'C'.

Early diagnosis of meningitis is essential to initiate appropriate intervention for the patient as well as the community. The pathogenicity, immunogenicity and epidemic capabilities differ according to the serogroup. Thus, the identification of the serogroup responsible of a sporadic case is crucial for epidemic containment.

Transmission is from person to person via droplets of respiratory or throat secretions. Close and prolonged contact (e.g. kissing, sneezing and coughing on someone, living in close quarters or dormitories (sharing eating or drinking utensils, etc) facilitate the spread of the disease. The incubation period is 4 days, ranging from 2 to 10 days.

N. meningitidis only infects humans; there are no animal reservoirs. Between 10 to 25% of the population are carriers of *N. meningitidis*, but the carriage rate may be much higher in epidemic situations.

Clinical case description

An illness with sudden onset of fever (>38.5°C rectal or >38.0°C axillary) and one or more of the following:

- Neck stiffness
- Altered consciousness
- Other meningeal signs (Kerning / Brudzinski sign positive)
- Petechial or purpurial rash
- Ongoing epidemic and epidemiological link to a confirmed case

In infants, suspect meningitis when fever is accompanied by bulging fontanelles.

Case classification

Probable: A case that meets the clinical case description, with

- Turbid CSF, OR
- Ongoing epidemic and epidemiological link to a confirmed case of meningitis

Confirmed: A suspect or probable case with laboratory confirmation:

- Positive CSF antigen detection, OR
- Positive culture from CSF or blood

DIPHTHERIA

Diphtheria is an acute infectious disease primarily of upper respiratory tract but occasionally of skin also. It is caused by toxigenic strains of *Corynebacterium diphtheriae*, which are gram positive, nonendospore forming pleomorphic rods. The dividing cells are folded together to form V and Y shaped figures resembling Chinese letters. Presence of polyphosphate metachromatic granules is a characteristic feature of this bacterium. With Albert's stain, the metachromatic granules stand out purple black against the green stained protoplasm.

There are three biotypes of this organism - *gravis*, *intermedius* and *mitis*. Recently, a fourth biotype - *belfanti* has also been described. Characteristically, *C. diphtheriae*

causes a typical form of infection which is manifested by a greyish-white membrane at the site of the localization of organisms. The disease begins with a sore throat and fever, followed by general malaise and swelling of the neck. This organism produces a powerful exotoxin capable of damaging the heart, kidney and muscles. Some toxigenic strains of *Corynebacterium ulcerans* can also cause diphtheria.

Though, the diagnosis of diphtheria is mainly clinical, laboratory is required to confirm the diagnosis, which is based on demonstration of the causative organism in the lesion. A presumptive diagnosis can be made on the basis of demonstration of the bacilli in the affected lesion by microscopic examination, but confirmation requires a positive culture.

Transmission is by direct person-to-person contact or by intimate respiratory and physical contact.

Clinical case description

An illness of the upper respiratory tract characterized by laryngitis or pharyngitis or tonsillitis and adherent membranes of tonsils, pharynx and / or nose

Case classification

- *Probable*: A case that meets the clinical case description
- *Confirmed*: A probable case that is laboratory confirmed or linked epidemiologically to a laboratory confirmed case

Laboratory diagnosis depends on isolation of toxin producing strain of *Corynebacterium diphtheriae* from membrane / lesion of a probable case.

Chapter 3

INVESTIGATION OF AN EPIDEMIC

An epidemic is defined as an “*unusual occurrence in a community or region of disease or, specific health related events clearly in excess of the expected occurrence*”.

The term *outbreak* is used to refer to a localized epidemic. The objectives of an epidemic investigation are:

1. To define the *magnitude* of the epidemic/outbreak in terms of time, place and person
2. To determine the *factors responsible* for the occurrence of the epidemic
3. To identify *cause, sources of infection and modes of transmission*, to determine measures necessary to control the epidemic
4. To make *recommendations to prevent future recurrences*

Steps in the investigation of an epidemic

1. *Verification of diagnosis*: this is the first step, and a quick verification will help confirm the diagnosis, and thus prevent misinterpretation of signs & symptoms by the lay public or a wrong diagnosis by any spurious laboratory reports. Laboratory investigations should be used to confirm the diagnosis wherever possible.
2. *Confirmation of existence of an epidemic*: This is done by comparing the disease frequencies during the same period of previous years in that particular population in the community. An arbitrary limit of two standard deviations is used to define the epidemic thresholds for common diseases such as influenza.
3. *Defining the population at risk*
 - a) Obtaining a map of the area: Before beginning any investigation it is necessary to have a detailed and current map of the area. The map should contain information regarding natural land marks, roads, and location of all dwelling units along the roads, or in isolated areas. The area may be divided into sections and all dwelling units be numbered in the sections.
 - b) Counting the population: Entire population or subgroups may be involved in the epidemic. However, it is prudent to undertake a complete census of the whole population by age and sex which will later on help to calculate the attack rates for that particular disease in various age groups. For this purpose lay health workers in sufficient numbers may be employed.

4. *Rapid search for all cases and their characteristics*
 - a) *Medical survey*: A medical survey should be carried out in the defined area to identify all cases including those who have not sought medical care. Ideally screening all members of the population will identify all the affected individuals. An epidemiological case sheet or questionnaire is used to collect all relevant data.
 - b) *Epidemiological case sheet*: The epidemiological case sheet/case interview form should be carefully designed to collect all relevant information. This includes: name, age, sex, occupation, social class, travel history, history of previous exposure, time of onset of disease, signs & symptoms of the illness, personal contacts at home, work/school, any special events like parties attended, food eaten, history of receiving injections or blood products. The information collected should be relevant to the disease under study.
In case of a large outbreak, it is not possible to interview all the cases, a random sample should be examined.
 - c) *Searching for more cases*: The patient may be asked about his/her contacts, and if they know about any similar cases. Cases admitted to local hospitals should also be taken into consideration. Search for new cases should be carried out everyday till the area is declared free of epidemic. This period is usually taken as twice the incubation period of the disease since the occurrence of the last case.

5. *Evaluation of ecological factors*: Ecological factors which have made the epidemic possible should be investigated. This includes sanitary status of eating establishments, water and milk supply, movements of human population, atmospheric changes such as temperature, humidity, air pollution etc. This will enable us to know the sources of infection, reservoirs and modes of transmission.

6. *Further investigation of population at risk*: To gather additional information about the population under study, further study in the form of medical examinations, examination of suspected food, faeces or blood, assessment of immunity status etc. should be done. The approach may be prospective or retrospective. Healthy individual may be studied in the case control fashion. This will allow classification of all members as per
 - a) exposure to specific potential vehicles
 - b) whether ill or not

7. *Data analysis*: The data collected should be analysed on ongoing basis, using epidemiological parameters - time, place and person. If the disease agent is known, the disease can be described based on the agent-host-environment model.
 - a) *Time*: Prepare a chronological distribution of dates of onset and construct an 'epidemic curve'. Look for time clustering of cases. An epidemic curve may suggest
 - i) A time relationship with exposure to suspected source.

- ii) Whether it is a common source or propagated epidemic.
- iii) Whether it is a seasonal or cyclic pattern suggestive of a particular infection.
- b) *Place*: Prepare a spot map (geographic distribution) of cases and if possible their relation to possible sources of infection e.g. water supply, air pollution, foods eaten, occupation etc. Clustering of cases may indicate a common source of infection.
- c) *Person*: Analyse the data by age, sex, occupation and other possible risk factors. Calculate attack rates/case fatality rates for those exposed and those not exposed.

8. *Formulation of hypothesis*: On the basis of time, place and person distribution or Agent-Host-Environment, formulate a hypothesis to explain the epidemic in terms of

- a) Possible source
- b) Causative agent
- c) Possible modes of spread
- d) Environmental factors which enable it to occur.

This tentative hypothesis should further guide the investigation.

9. *Testing of the hypothesis*: All reasonable hypotheses need to be considered and weighed by comparing the attack rates in various groups for those exposed and those not exposed. This will help to ascertain whether the hypothesis is consistent with all the known facts or not.

10. *Writing the report*: The report should be complete. Information to be included in the final report include

- a) *Background*:
 - Geographical location
 - Climatic conditions
 - Demographic status
 - Socioeconomic situation
 - Organization of health services
 - Surveillance, etc.
- b) *Historical data*: Previous occurrence of epidemics, occurrence of related disease, etc.
- c) *Methodology of investigation*: This includes case definition, questionnaires used in survey, survey teams, type of survey, collection of any laboratory specimens, etc.
- d) *Analysis of data*: This includes analysis of clinical data, epidemiological data, modes of transmission, laboratory data and finally involves the interpretation of data.
- e) *Control measures*: Definition of strategies and methodology of implementation, evaluation of control measures.

Chapter 4

COLLECTION, TRANSPORT & STORAGE OF SPECIMENS

The appropriate selection, collection, rapid transport and processing of specimens is very important for microbiologic diagnosis. When sample collection and management are not priorities the laboratory can contribute little to patient care / community health. Consequently all members of the staff involved in the process must ensure the critical nature of ensuring quality. It is the responsibility of the laboratory to provide complete and accurate specimen management information to the health care workers who have the primary responsibility of collecting the specimens. The information provided should address safety, selection, collection, storage, labelling, transportation and acceptability.

Safety

Biosafety in the laboratory is of primary concern to the laboratories. Healthcare workers may be unaware of potential etiologic agents residing in the specimen being transported to the laboratory. To protect the safety of the healthcare worker collecting the sample and laboratory personnel, the following precautions must be followed when collecting specimens:

- During specimen collection wear personal protective equipment such as gloves, laboratory coat and where appropriate, a mask and / or goggles.
- Use leak-proof specimen containers and transport bags that have a separate outside compartment for the test requisition form.
- Never transport syringes with needles to the laboratory. Instead, transfer the contents to a sterile tube or remove the needle with a protective device, recap the syringe, and place it in a sealable, leak proof plastic bag.
- Make sure screw-cap lids are fastened evenly and securely. Ensure that no label material is caught in the threads of the lid.
- Do not transport leaking containers to the laboratory because test results will be compromised and it is a hazard to couriers and laboratory personnel.
- To protect the safety of others, take care not to contaminate the outside of the specimen container or the laboratory requisition form.

General specimen selection and collection guidelines:

- Wash hands before and after the collection.
- Aseptic techniques must be employed during collection to prevent the introduction of micro-organisms into the patient's anatomical space, and to prevent the sample from being contaminated during collection.
- Avoid contamination from indigenous flora, whenever possible, to ensure a sample representative of the infectious process.
- Collect specimens before the administration of antimicrobial agents.

- Collect the specimen at the appropriate stage of disease.
- Make certain that the specimen is representative of the disease.
- Select the correct anatomic site from which to obtain the specimen, and collect the specimen by the recommended technique.
- Collect adequate volume, as insufficient material may yield false negative results.
- Collect the specimen in an appropriate screw capped, external threaded, unbreakable, leak-proof container.
- Ensure that the outside of the specimen container is clean and uncontaminated.
- Close the container tightly so that its contents do not leak during transportation.
- Label the container appropriately and complete the requisition form.
- Arrange for immediate transportation of the specimen to the laboratory

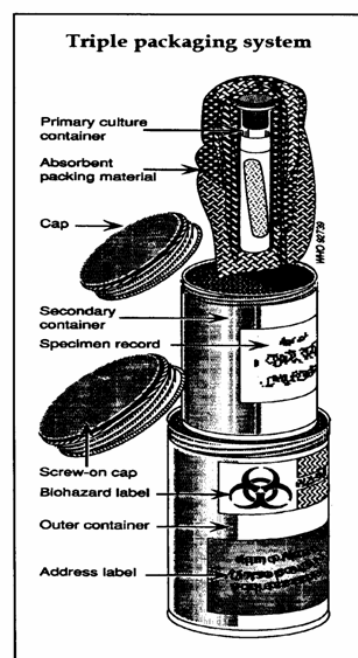
Table 4.1 – Specimens to be collected

Probable diagnosis	Trigger event	Sample collection / storage / transport
Acute watery diarrhoea	A single case of cholera or epidemiologically linked case of diarrhoea / a case of severe dehydration or death due to diarrhoea in a patient >5 yrs old Clustering of cases particularly village or ward where >10 houses having at least 1 case of loose stools irrespective of age	Collect freshly passed stool in a clean, wide mouthed container. Ideally collect within 48 hours of onset of diarrhoea Transport stool specimen quickly to the laboratory, in case of delay store at 4-8°C for 48 hrs and transport in Cary Blair medium. In case of delay beyond 2 days store in freezer at -20°C
Typhoid	More than 30 cases of prolonged fever a week from the entire PHC or 5 or more cases per week from 1 sub centre OR More than 2 cases from a single village/urban ward with 1000 population	Blood sample is collected for culture & serology For <u>blood culture</u> collect the required volume of venous blood aseptically (5-10 ml for adults, 1-2 ml for children) and transfer carefully to blood culture bottle containing TSB broth. Transfer immediately to lab, taking care to avoid spillage. In case of delay store in an incubator at 37°C for up to 24 hrs For <u>serology</u> collect a paired serum sample - first sample early in the disease preferably within 3 days & second sample 10-15 days later
Jaundice	Clustering of cases from a particular village/urban ward where more than 2 cases of jaundice in different households or more than 10 cases per PHC per week	Collect blood sample for etiologic diagnosis of the causative agent of jaundice Separate serum sample for detection of antibodies to HAV & HEV
Measles	A single case of probable measles from a tribal or remote area, or two or more cases with fever with rash	The virus can be isolated in the early stages of the disease from upper respiratory tract specimens like nasopharyngeal aspirate Blood is collected for detection of specific IgM antibodies
Dengue DF/DHF	Even a single case of suspected DHF from a community Rising number of fever cases for previous 3 weeks	Paired serum samples are collected to demonstrate rising levels of anti-dengue antibodies
Meningitis	2 cases with fever with altered consciousness / seizures	CSF is to be collected by a trained physician or a medical officer Collect about 1-2 ml CSF in each of the 3 collection vials for cell counts, biochemical & microbiological investigations Do Gram staining, culture and antigen detection from the microbiological specimen CSF for bacteriology should never be frozen, transported or stored at room temperature - store in an incubator, if available. CSF for virus detection can be stored at 4-8°C Collect blood sample for culture

Storage & transportation of specimens

In general samples should be kept at 2-8°C during storage and transport except the CSF samples collected from cases of pyogenic meningitis, which can be stored at room temperature or in the incubator. The quality of sample can deteriorate during storage or transportation which affects the diagnostic results. Hence, special care should be taken during transport of samples to the laboratory to protect them from heating or drying.

- All specimens must be promptly transported to the laboratory, preferably within 2 hours.
- Specimens for bacterial culture should not be stored for more than 24 hours before transport to the laboratory.
- Specimens suspected to yield environmentally sensitive organisms as *Streptococcus pneumoniae*, *Neisseria sp.* and *Haemophilus influenzae* (which are sensitive to cold temperatures) should not be refrigerated.
- Specimen containers relating to single case investigation should be placed in a plastic bag with an absorbent material surrounding the specimen so that even if the whole specimen leaks out, it will be absorbed.
- The lab report form should be sealed within a separate plastic bag and wrapped round the specimen or attached firmly to box of specimens.
- The material should be packed in an insulated carton / carrier to transport a specimen to the lab.
- All specimens should be considered as potentially pathogenic and accordingly labelled with internationally accepted biohazard label.
- Avoid repeated thawing & freezing of specimens.
- For transportation it should be placed in a *triple container system*:
 - *Primary container* which has the specimen and is leak proof with a screw cap.
 - *Secondary container* made of durable, waterproof material with a screw cap, with an absorbent material surrounding the specimen so that even if whole specimen leaks out, it will be absorbed.
 - *Tertiary container* is usually made of wood, plastic or cardboard. It should be capable of withstanding the shocks and trauma of transportation. Dry ice can be kept between this and the secondary container along with sufficient absorbents.
- As an alternative, follow triple packaging method described in the “Manual of Laboratory Techniques for District Public Health Laboratories”.



Specimen acceptability or rejection criteria

At times, specimens arriving in the laboratory may have been improperly selected, collected or transported. Processing and reporting such specimens may provide misleading information that can lead to misdiagnosis and inappropriate therapy. Consequently, the laboratory must adhere to a strict policy of specimen acceptance and rejection.

The following are some examples under which samples could be rejected in the laboratory:

- No label
- Improper or leaking container
- Prolonged transport
- Insufficient quantity
- Specimen collected in an inappropriate container
- Contamination suspected
- Inappropriate transport or storage
- Haemolysed / lipemic serum sample

In all the above cases, immediately contact the submitting health care worker. For specimens collected by non invasive means, have a new specimen submitted, but for invasive specimens or for other samples which cannot be collected again, process the specimen only after consulting the person who obtained the specimen. Note the problem on the form and report.

Labelling and identification of specimens

Proper identification of every patient sample is as important as the quality of the sample.

Each patient should be given a unique identification number. This unique identification number and the patient name should be present on all specimens, epidemiological data forms, and the laboratory transmittal forms and used as a common reference.

The sample should be labelled using pre-printed labels / glass-marking pencil / permanent markers / adhesive tape, etc. Labels should be firmly affixed to the specimen container. It should contain the following:

- Patient's name
- Identification number
- Specimen type
- Date & time of collection

Glass slides for microscopy must be labelled individually, using glass marking pencil. This should not interfere with the staining process. Each slide should bear:

- Patient's name
- Identification number
- Date of collection

Collection of specimens

Some of the important specimens and their proper collection and transportation methods are described here so as to ensure quality.

1. Blood specimens

Blood and separated serum are the most common specimens taken to investigate outbreaks of communicable disease. Venous blood can be used for isolation and identification of the pathogen in culture, serum for the detection of specific antibodies, antigens, or toxins (e.g. by ELISA/other serological assay). For the processing of most specimens for diagnosis of viral pathogens, serum is preferable to whole blood except where otherwise directed. When specific antibodies are being assayed, it is often helpful to collect paired sera, i.e. an acute sample at the onset of illness and a convalescent sample one to four weeks later. Blood can also be collected by finger prick for the preparation of slides for microscopy or for absorption onto special filter paper discs for analysis. Whenever possible, blood specimens for culture should be taken before antibiotics are administered to the patient.

Venous blood samples

Materials for collection:

- Skin disinfection: 70% alcohol (isopropyl alcohol, ethanol) or 10% povidone iodine, swabs, gauze pads, band aid
- Disposable gloves
- Tourniquet, disposable syringes and needles
- Sterile screw-cap tubes for collecting blood
- Sterile screw cap tubes for storage of separated serum
- Blood culture bottles (50ml for adults, 25ml for children) with appropriate media for identification of bacterial pathogen by culture method
- Labels and marker pen

Performing venepuncture for blood culture

- Label the collection container before commencement of venepuncture
- Wear gloves
- Use sterilized / sterile disposable syringes and needles
- Apply a tourniquet and palpate arm for suitable vein
- After the vessel site is selected, a 5 cm area of skin should be disinfected with 70% alcohol by swabbing concentrically outward from the venepuncture site
- The site should be cleansed once again, with 10% povidone-iodine, again in a concentric motion, from inside outwards.
- Allow the iodine to dry completely before performing venepuncture (this should take 1 - 2 minutes)
- Prepare the blood culture bottles by applying labels and cleaning the tops with 70% alcohol before starting the procedure
- 5-10ml of blood should be withdrawn from the puncture site for one bottle

- Inoculate the blood culture bottle/s. Do not change needles between venepuncture and inoculation of the bottles, or between bottles. The risk of needle-stick injury is increased, while the chance of contamination is not significantly lessened
- Discard needle and syringe in an appropriate manner
- Clean the top of the blood culture bottles with an alcohol wipe to remove any residual blood
- Invert bottles so that clots will not form

Table 4.2 – Volume of blood to be collected at different ages

Age	Ideal volume in each of 2 bottles
< 2 years	1 ml
2-10 years	5 ml
>10 years	10 ml

Blood for serological testing¹

- Using the materials and methods described above, draw 5-10 ml of venous blood and transfer to a screw cap tube without anti-coagulant
- The vial for collecting blood should be sterile, dry and properly labelled
- After drawing blood, the needle should be removed from the syringe before transferring blood from syringe to the sterile tube
- Let the specimen clot for 30 minutes at ambient temperature; do not shake
- Then place in a cool box for clot retraction at 4-8°C, for a minimum of 1-2 hours
- If facilities for separation of serum are not available, then it should be refrigerated at 4°C (not frozen)
- Otherwise centrifuge @1500rpm for 10 minutes (ensure that the centrifuge is in good condition and the tubes are properly closed and balanced to avoid breakage and spilling)
- Separate the serum aseptically from the clot using a disposable transfer pipette
- Transfer equally to 2 plastic screw cap tubes
- Secure the caps tightly



Separation of serum from clotted blood sample

Handling and transport

- If serum will be required for testing, separation from blood should take place as soon as possible, preferably within 24 hours at ambient temperature

¹ In case blood cultures are also collected simultaneously, the blood culture bottles should be inoculated first.

- If the specimen will not reach a laboratory for processing within 24 hours, serum should be separated from blood prior to transportation
- Sera may be stored at 4-8°C for up to 10 days
- If testing is delayed for a long period, serum samples may be frozen
- If separation on site is not possible, or is inadvisable for safety reasons, the blood sample should be stored at 4-8°C (whole blood samples should not be frozen)
- Protect such samples from excessive vibration while transporting

2. Cerebrospinal fluid (CSF) specimen

The specimen must be taken by a physician experienced in the procedure. CSF is used for diagnosis of viral, bacterial, parasitic, and fungal meningitis.

Handling and transport

- CSF is collected in properly labelled sterile screw-cap tubes.
- Specimens should be delivered to the laboratory and processed as soon as possible.
- If the samples cannot be promptly transported, separate tubes should be collected for bacterial and viral processing.
- CSF specimens for bacteriology are transported at ambient temperature
- CSF should never be refrigerated for bacterial culture (bacteria like *N. meningitides*, *Strept. pneumoniae* and *H. influenzae* are thermo-labile)
- CSF specimens for virology do not need transport medium (they may be transported at 4-8°C)

3. Faecal specimen

Faecal specimens may be collected in the early stages of a disease, when pathogens are likely to be present in the stool in high numbers i.e. soon after onset of diarrhoea (for viruses <48 hours and for bacteria <4 days), and preferably before the initiation of antibiotic therapy.

- Stool specimens should be collected in a wide-mouthed sterile container with a leak-proof screw-capped lid.
- The collected stool should be processed as soon as possible upon receipt in the laboratory.
- In case of delayed transport (> 2 hours), collect a small amount of stool on a swab and inoculate Cary-Blair transport medium.
- Stool is the preferred specimen for culture in a case of diarrhoea.
- Rectal swab may be collected for acutely ill patients, newborns or when stool specimen is not available. It is advisable to collect two swabs from each patient, one for microscopy and other for bacterial/viral studies.

Materials required

- Sterile wide mouth, external threaded, unbreakable, leak proof container

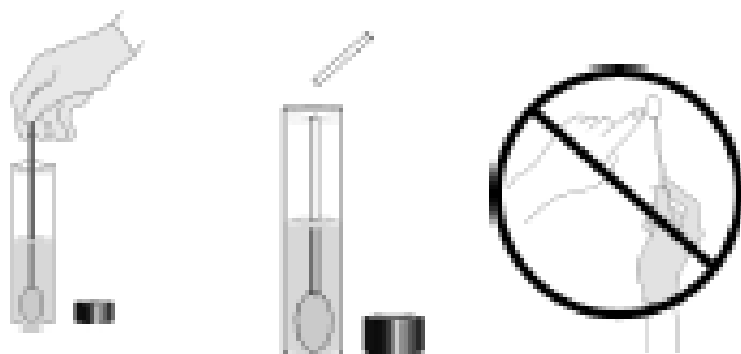
- Spatula for transferring the specimen to the container
- Transport medium (e.g. Cary Blair's) if delay in transport is suspected

Collection and Transport of Stool Samples

- Label the specimen container clearly with patients name and date of collection
- Collect freshly passed liquid stool.
- Place a separate clean container with a wide opening, or plastic wrap in the toilet bowl and transfer enough faeces with spatula to at least half fill the specimen container. Collection of stools from bed-pans is not recommended
- Alternatively, collect a rectal swab by using a sterile cotton tipped swab
- Send the sample to the laboratory in a tightly sealed screw capped sterile bottle, in a sealed bag
- If delay of more than two hours is anticipated, swab the stool sample collected and put the swabs into Cary-Blair medium (for bacterial pathogens) - should reach laboratory in 2-3 days time; can keep at RT
- Preserve/Transport collected sample at room temperature if cholera is suspected, otherwise transport in cold conditions

Collection of rectal swab

- Moisten 2 swabs in an appropriate transport medium (e.g. Cary-Blair).
- Insert swab 2-3 cm into rectum and gently rotate.
- Gently rotate up to 90 degrees, so that faeces covers the swab.
- Withdraw the swab.
- After collection of both swabs (one at a time), place both swabs into the same tube deep enough so that medium covers the cotton tips.
- Break off top portion of sticks and replace the cap
- Label the specimen and place it in a plastic bag with the appropriate request slip.



Inoculation of rectal swab into Cary Blair's medium

4. Respiratory tract specimen

Specimens are collected from the upper or lower respiratory tract, depending on the site of infection. Upper respiratory tract pathogens (viral and bacterial) are found in

throat and nasopharyngeal specimens. Lower respiratory tract pathogens are found in sputum specimens. When acute epiglottitis is suspected, no attempt should be made to take throat or pharyngeal specimens since these procedures may precipitate respiratory obstruction.

Materials Required

- Transport media – bacterial / viral
- Dacron and cotton swabs
- Tongue depressor
- Flexible wire calcium alginate tipped swab (for suspected pertussis)
- Nasal speculum (for suspected pertussis – not essential)
- Suction apparatus or 20-50 ml syringe
- Sterile screw-cap tubes, and wide-mouthed clean sterile jars (minimum volume 25ml)

Upper respiratory tract specimens - Throat swab

- Hold the tongue down with the depressor
- Locate areas of inflammation and exudate in the posterior pharynx and the tonsillar region of the throat behind the uvula using a good light source
- Rub the area back and forth with a Dacron or calcium alginate swab
- Withdraw the swab without touching cheeks, teeth or gums and insert into a screw-cap tube containing transport medium
- Break off the top part of the stick without touching the tube and tighten the screw cap firmly
- Label the specimen containers

Lower respiratory tract specimens - Sputum

- Collect an early morning sample after rinsing the mouth with water
- Instruct the patient to inhale deeply 2-3 times, cough up deeply from the chest and spit in the sputum container by bringing it closer to the mouth
- This should be done in the open or away from other people
- Make sure the sputum sample is of good quality - a good sputum sample is thick purulent and sufficient in amount
- Avoid saliva or postnasal discharge
- Minimum volume should be about 1 ml
- Label the specimen containers

Handling and transport

- All respiratory specimens except sputum are transported in appropriate bacterial / viral transport media
- Transport as quickly as possible to the laboratory to reduce overgrowth by commensal oral flora
- For transit periods up to 24 hours, transport bacterial specimens at ambient temperature and viruses at 4-8°C in appropriate media

Chapter 5

STANDARD OPERATIVE PROCEDURES

CHOLERA CULTURE

Equipments & Supplies

- Glass slides: 75 x 25 mm size, 1.35 mm thick, 50 slides / box
- Coverslips: 18 x 18 mm size, no 1 size; 10 g (about 50 coverslips)/packet, 20 packets / box
- Amber coloured storage bottles
- Rectal swab: made up of absorbent cotton wool, sterilized

Related material

- Dehydrated culture media like TCBS, MacConkey agar
- Transport media - Alkaline peptone water / Cary Blair media
- Disposable petri plates, 88-90 mm diameter, polypropylene, individually wrapped, pre-sterilized and disposable
- Antisera - *Vibrio cholerae* antisera, poly 01 & 0139-Bengal
- Accessories - loop holder, nichrome wire, discarding jar with disinfectant

Processing of Sample

Macroscopy - Gross examination should be recorded for all samples.

- Colour
- Consistency - solid (S), semi formed (SF), or watery
- Abnormal constituents - blood, mucus, worms, etc.

Microscopy

- *Wet mount*: Put one drop saline along with a loopful of the sample in the centre of a glass slide and put a cover slip; examine under 10X/40X objective of a microscope. Look for pus cells, RBCs, trophozoites, etc. Experienced observer can also recognise darting motility in this preparation.
- *Hanging drop*: In case of watery sample, place a loop of plasticine on the coverslip and then place a drop of the stool sample on the cover slip at the centre of the plasticine loop. Invert the cover slip over a slide. Examine the edge of the drop under high power to look for darting motility suggestive of *Vibrio cholerae*.

Culture

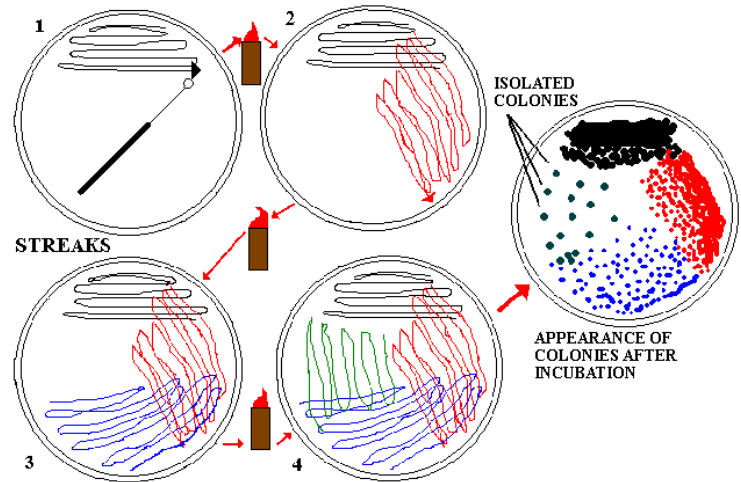
The following media are used routinely

- MacConkey agar
- TCBS (Thiosulphate Citrate Bile Salt agar): selective medium used for isolation of *Vibrio cholerae*

- Alkaline Peptone water: Selective enrichment media specially for the isolation of *Vibrio cholerae* (subculture on MacConkey and TCBS after 6 hours of incubation from the surface pellicle, without shaking)

Methods for *Vibrio* isolation

- For stool culture preferably use bio safety level 2 cabinet along with all aseptic precautions including gloves and masks
- Select fresh culture plates (preferably < 7 days old)
- Dry culture plates at 37°C incubator for about half an hour



Plating technique for getting isolates colonies on solid media

- Label all culture plates and liquid media tubes and arrange them
- Label all the plates
- Take a loopful of stool specimen with Nichrome wire loop & make a primary well on MacConkey agar plate & TCBS
- Sterilize Nichrome loop in blue portion of Bunsen burner flame, and cool the loop
- Do primary streaking, followed by heat sterilization / cooling of the loop; re-streak in parallel lines so as to isolate separate single colonies as far as possible
- Incubate at 37°C incubator for 24 hours
- Next day observe colony morphology on media plate
- Also, inoculate enrichment broth (APW) after labelling the same
- Incubate the inoculated APW tube at 37°C and subculture on to TCBS media within 4 to 6 hours on incubation

Cultural characteristics

- MacConkey Agar: After 18-24 hours at 37°C, suspected vibrio like colonies are pale or nearly colourless, 1-3mm in diameter, moist, flat, circular and smooth. These are catalase and oxidase positive.
- TCBS: After 18-24 hours at 37°C, the colonies are flat, yellow and shiny. These are oxidase positive.



Colonies of *V. cholerae* on TCBS medium

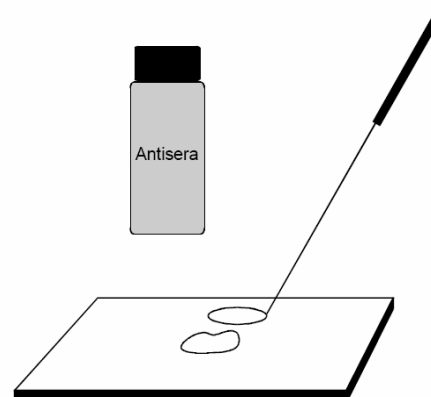
- Subculture the suspect colonies on Nutrient agar (NA) or Mueller Hinton (MH) Agar for reconfirmation of oxidase test and performing the slide agglutination test using *Vibrio cholerae* antiserum.
- Stock all isolates in good quality nutrient agar



Oxidase test (positive)

Identification tests

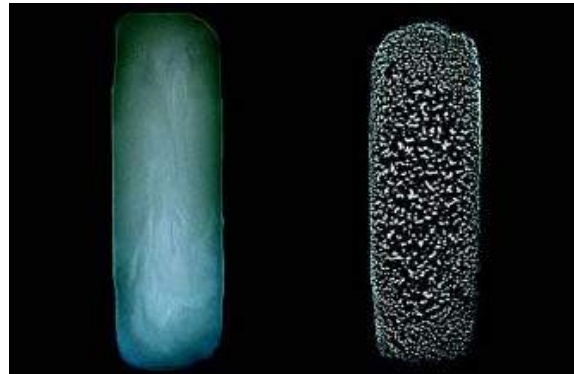
- Gram stain (for details refer annexure 5)
 - Place a drop of normal saline on a labelled, clean glass slide and emulsify a small portion of suspected vibrio like colony
 - Once the smear is dry, heat fix it and stain
 - On examination under oil immersion 100X objective small, curved gram negative (GN) rods will be seen
- Oxidase Test (for details refer annexure 7)
 - Pick a speck of colony (from MacConkey agar / TCBS) using a glass rod & smear it on the filter paper
 - Development of purple colour within 30 seconds is indicative of positive oxidase reaction
 - Check reagent using positive and negative controls
- Slide agglutination test for confirmation of *Vibrio cholerae* (using poly O1 and O139 antisera)
 - Inoculate suspected vibrio looking colony on to non-selective agar (NA/MH) and incubated at 37°C for 24 hrs; the growth obtained is then used for slide agglutination test
 - Take out cholera antisera from the refrigerator and bring it to room temperature
 - For carrying out slide agglutination test, take 2-3 clean glass slides
 - Make them grease free by cleaning with cotton & then passing over flame several times; cool the slide
 - All sera manipulation are to be done in an aseptic manner in clean area so as to prevent antisera contamination
 - Take two drops of normal saline with Nichrome wire loop and put over a clean glass slide - side by side
 - Pick up a suspected colony with a straight wire and emulsify in both the saline drops one by one
 - Check for auto-agglutinability i.e. formation of coarse



A loop may be used to make a suspension for slide agglutination tests

granules without the addition of antisera

- In case of rough strain, auto-agglutinability will be observed - in such a case, no further serotyping should be attempted
- In case the strain is smooth & forming a smooth suspension, add a drop (small) of cholera antiserum (Poly O1)
- Look for clumping i.e. formation of coarse granules which indicate agglutination
- In case there is no agglutination with Poly O1 *V. cholerae* antisera, repeat the steps with O139-Bengal antisera
- Periodically check antisera with known *Vibrio cholerae* O1 and O 139 strains
- Inform laboratory incharge for further action or public health measures in case cholera is diagnosed at district level laboratories



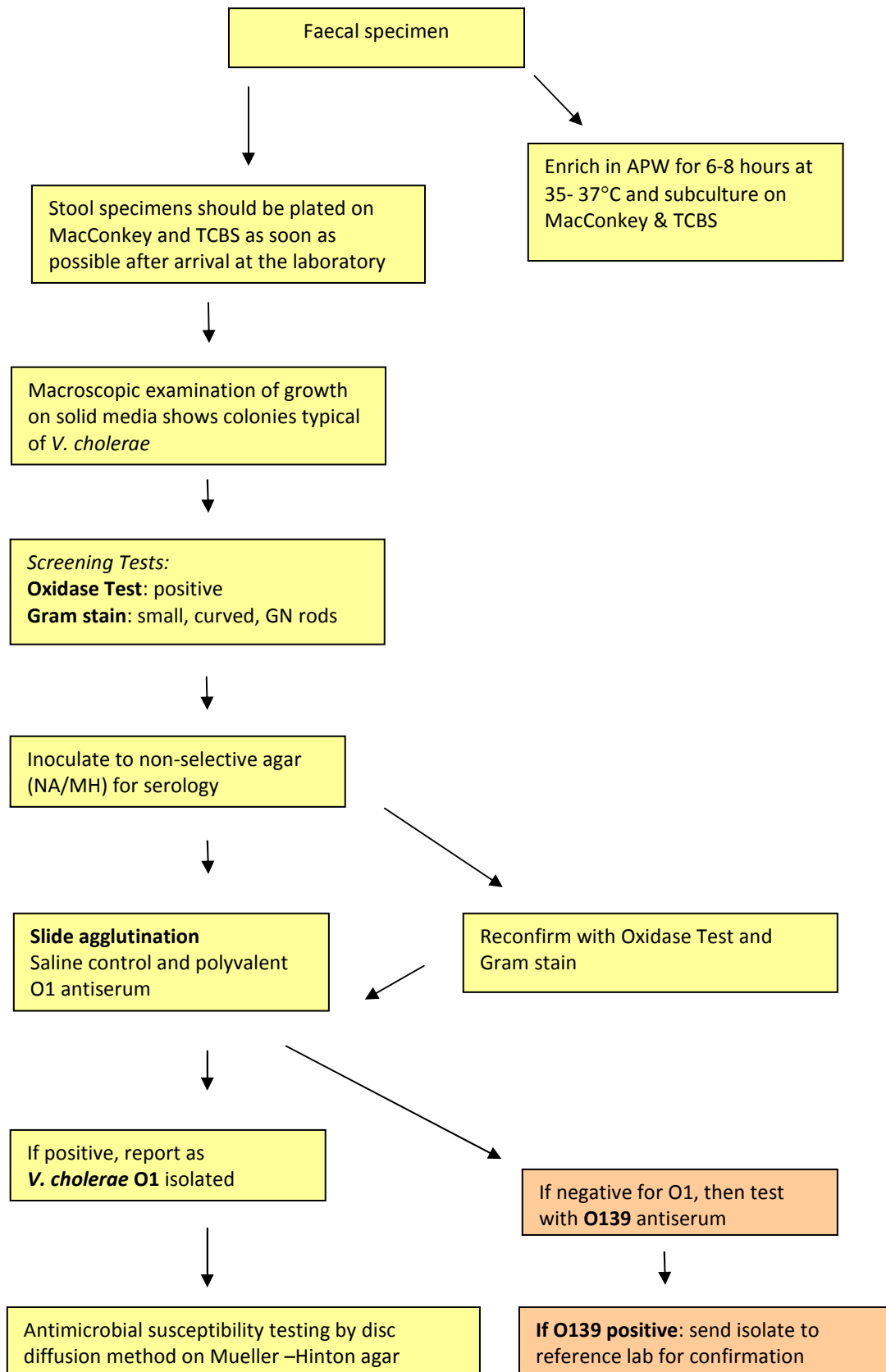
Saline control (left) ensures validity of the test; agglutination seen on the right

Antimicrobial susceptibility testing

The stocked strains of *Vibrio cholerae* must be sent to the referral lab for antimicrobial susceptibility testing.

The isolates can be referred for re-confirmation with detailed characterization and epidemiological analysis.

Flowchart for processing stool for Cholera



TYPHOID CULTURE

Blood, stool and urine are the clinical samples from which the causative agent of typhoid can be isolated. However, the isolation rate is different at different stages of disease. The best isolation rates from blood have been seen in the first week of illness, with faeces in the second week and with the urine in the third week of illness. Two serum samples are collected at an interval of at least 10-14 days to demonstrate a four fold increase in antibody titre.

Blood culture

Out of the many clinical samples that can be used for culture, blood culture is the most informative. Blood samples of suspected septicaemia patients are collected before the first dose of antibiotic is given. There shouldn't be any delay in transferring the sample into the bottles to get a better yield. The organisms will soon be exposed to the nutritive medium which facilitates their growth and also the effect of any antibiotic or the antibodies of the host will be reduced because they get diluted in the broth. TSB broth should contain sodium polyanethol sulfonate (SPS). Liquoid / SPS is an anticoagulant that inhibits serum bactericidal activity, and inactivates lysozymes and aminoglycosides.

Reagents

- Blood culture bottles, containing 25ml (paediatric) 50 ml (adult) of Tryptic Soya broth (TSB) containing SPS
- Culture Media: Blood Agar, MacConkey Agar

Procedure

- Collect blood sample aseptically, as described in chapter 4.
- Send the bottles to the laboratory immediately (do not refrigerate)
- The blood culture bottles are shaken gently for proper mixing and aeration, and incubated at 37°C; after 24 hours of incubation inspect for any signs of microbial growth without shaking
- If there is a layer of sedimented red blood cells at the bottom covered by transparent pale yellow broth culture, it is considered to be sterile
- All blood culture bottles are subcultured on Blood agar & MacConkey agar after 24 hours and 48 hours of incubation, irrespective of turbidity

All negative samples are incubated for seven days. Before discarding, a final subculture is done and findings are recorded.

Media	Length of incubation (hours)
TSB broth	7 days – subculture after 24 & 48 hours, or when turbidity appears
Blood agar	24 & 48
MacConkey	24 & 48

Cultural characteristics

- MacConkey Agar: After 18-24 hours at 37°C, the colonies are pale yellow or nearly colourless, 1-3mm in diameter, moist, circular and smooth convex surface

Biochemical reactions (*S. typhi* / *S. paratyphi A*) (for details refer to Annexure 6 & 7)

- Catalase – positive
- Oxidase – negative
- Indole test – negative
- Urease – negative
- Citrate – positive (*S. typhi*) & negative (*S. paratyphi A*)
- TSI (Triple sugar iron)

Species	Slant	Butt		H ₂ S
	Lactose / Sucrose	Glucose	Gas	
<i>S. typhi</i>	- (pink)	+ (yellow)	-	+ (trace)
<i>S. paratyphi A</i>	- (pink)	+ (yellow)	+	-

Slide agglutination

If on the basis of biochemical reactions, the organism has been identified as Salmonella, its identity can be confirmed with slide agglutination test, using polyvalent 'O' and polyvalent 'H' antisera against Salmonella. For group identification, monovalent 'O' specific antiserum for *S. typhi* is factor 9 and for *S. paratyphi A* is factor 2.

- Take out antisera from the refrigerator and bring them to room temperature.
- Take clean glass slides & make them grease free by passing over flame several times; cool the slide
- Take two drops of normal saline with Nichrome wire loop and put on sides of a clean glass slide
- Pick up a suspected colony with a straight wire and emulsify in both the saline drops one by one so as to obtain uniform suspensions
- Check for auto-agglutinability i.e. formation of coarse granules without the addition of antisera
- In case auto-agglutinability is observed, no further serotyping should be attempted
- In case the strain is smooth (forming a smooth suspension), add a drop (small) of polyvalent 'O' antiserum against salmonella
- Rotate slide for one minute and look for clumping i.e. formation of coarse granules which indicate agglutination
- If this gives visible agglutination within two minutes, the process is repeated with polyvalent 'H' antiserum and then with factor 9 antiserum
- Precaution - all sera manipulation are to be done in an aseptic manner in clean area so as to prevent antisera contamination

Antimicrobial susceptibility testing

The emergence and spread of *S. typhi* resistant to multiple antibiotics has assumed a massive public health problem. Multi-drug resistant typhoid fever has become endemic in many developing countries. Thus conventional antibiotics such as chloramphenicol, ampicillin and cotrimoxazole can no longer be considered as the

first line drugs in the treatment of typhoid fever. Each and every isolate of *S. typhi* should be subjected to antimicrobial susceptibility testing, as it will help in the management of the patient more efficiently and also in generating surveillance data which will be useful to formulate an antibiotic policy in a given hospital.

Stool culture

- Stool culture should be attempted in second or third week
- Rectal swabs give inferior results than faeces culture
- Culture on XLD agar gives good results in isolating salmonella from faeces

TYPHI-DOT TEST FOR TYPHOID

Using TyphiDot test, diagnosis of fever caused by *Salmonella typhi* can be made in a short time (within 1 hour of collecting the sample). The test can be performed using a single sample of blood / serum. The test can be easily performed in a district laboratory without the need of any sophisticated equipments. The presence of IgM and IgG antibodies made against a specific antigen on the outer membrane of *Salmonella typhi* are detected by incubating nitrocellulose strips dotted with the specific antigen protein with the patient's sera and control sera. To visualise the antigen-antibody complex, the strips are simultaneously incubated with peroxidase-conjugated antihuman IgM & IgG upon addition of the chromogenic substrate, the results can be read visually. Positive reading is indicated by the blue colour as intense as or more intense than that of the positive control.

Reagents and material provided with the kit

TyphiDot contains reagents and antigens dotted strips for detection of specific IgM and IgG antibodies to *Salmonella typhi*, inclusive of controls

- Use of sterile disposable pipette tips is recommended
- Do not use kit beyond expiration date and do not mix reagents from different batch numbers

Contents	28 Tests	56 Tests
Predotted antigen strips	56 Strips	112 Strips
Samples diluent (A1)	15 ml	30 ml
Washing buffer (10x) (A2)	10 ml	20 ml
Prediluted anti-Human IgM *HRP (B1)	7.3 ml	14.6 ml
Prediluted Anti-Human IgG *HRP (B2)	7.3 ml	14.6 ml
Colour reagents Substrate A (C1)	5.5 ml	11 ml
Substrate B (C2)	15.5 ml	31 ml
Positive control	60 ul	120 ul
Negative control	60 ul	120 ul
Worksheet	2	4

Note: Store kit at 2-8°C. Allow kit to warm to room temperature (minimum 23°C) before doing the test.

Materials required but not provided

- Measuring cylinder (100ml)
- Micropipettes (2-20 & 1000 ul)
- Sterile micropipette tips
- Forceps
- Wash bottles
- Filter paper
- Distilled water
- Gloves
- Discarding jar containing suitable disinfectant
- Aluminium foil
- Conical flask covered with aluminium foil or a dark reagent bottle

Preparation of Reagents

- Washing buffer (10x)
 - Dilute washing buffer into 90 ml of distilled water to a final concentration of 1x
 - The diluted washing buffer is sufficient for the entire kit
 - Store it separately in a clean bottle at 2-8°C and use when necessary

10X (A2)	Distilled water	1X
10 ml	90ml	100ml

- Colour Development Reagents
 - Prepare 30 minutes before use
 - Substrate A (C1) and substrate B (C2) should be brought to room temperature before mixing - avoid exposing these reagents to strong light during incubation or storage
 - Based on the number of tests (including controls), add the recommended volume of substrate B into a reagent bottle of a flask covered with aluminium foil
 - Then add the recommended volume of substrate A and mix well, as given below:

No. of tests (including controls)	3	6	9	12	15	18	21	24	27	30
Substrate A (in ml)	0.25	0.5	0.75	1.0	1.25	1.5	1.75	2.0	2.25	2.5
Substrate B (in ml)	1.25	2.5	3.75	5.0	6.25	7.5	8.75	10	11.25	12.5






Procedure

1. Divide the reaction tray into columns. Mark one column as 'M' and another column as 'G'
2. Using forceps, remove the predotted antigen strips and place them with the marked side up onto a filter paper
3. Align all antigen strips with the marked side on your right
4. Using a ball-point pen, for each serum sample, label one strip as M and other as G

5. Perform similarly for positive and negative controls
(Make sure that there is an IgM and an IgG strips (2 strips) for each patient or control serum)
6. Add 250 µl of sample diluent into the appropriate 'M' and 'G' reaction wells
7. Place the 'M' or 'G' strips into the appropriate reaction wells
8. Shake tray gently to allow strips to be thoroughly wet
9. To the appropriate 'M' or 'G' reaction well, add 2.5 µl of either control or test serum to achieve a final serum dilution of 1:100
10. Gently aspirate the solutions
11. To avoid cross-contamination, use a new sterile disposable pipette tip for each specimen
12. Make sure all membranes are with the marked side up, fully immersed in the first antibody solution
13. Incubate at room temperature on a rocker platform (optional) for 20 minutes
14. Shake the tray gently, every 5-10 minutes if a rocker platform is not available
15. Aspirate the first antibody solution into a discard jar containing a disinfectant
16. Add 250 µl of prepared washing buffer into each well and wash 3 times, each for five minutes
17. Using sterile micropipettes tips, add 250 µl of prediluted anti-Human IgM (B1) into the 'M' well and 250 µl of prediluted anti-Human IgG (B2) into the 'G' well
18. Cover the tray with aluminium foil and incubate for 15 minutes at room temperature (min 23°C) on a rocker platform
19. Aspirate the second antibody solution and wash 3 times for 15 minutes as described earlier
20. Add 250 µl of the colour development solution into each well
21. Cover the tray and incubator on the rocker platform
22. Allow 15 minutes for colour development
23. Stop the reaction by aspirating the solution and briefly rinsing the strips in distilled water (3times)
24. Place similarly coded strips for IgM and IgG for each patient and test control onto filter paper to dry - interpret the results (see guided interpretation charts)
If interpretation cannot be done immediately, store the strips submerged in distilled water for up to 1 day
25. After use, rinse the tray thoroughly with distilled water
26. Store dry for re-use
27. For a permanent record, paste the dried membranes in the relevant position on the worksheet provided

Interpretation

Basic Principles - To interpret the TyphiDot the colour intensity of the dots produced by the test sera must be equivalent to or greater than those of the positive control. Reading TyphiDot result depends entirely on your observation of the intensity of each dotted antigens after colour development. There are few very important principles to remember.

SAMPLES	RESULTS	INTERPRETATION
Positive controls		
Sample 1.		POSITIVE
Sample 2.		NEGATIVE
Sample 3.		INCONCLUSIVE READ AS NEGATIVE.
Sample 4.		INCONCLUSIVE READ AS NEGATIVE

- Always compare each IgM test with that of the positive IgM control strip. Similarly, compare the IgG test strips with that of the IgG positive control strips.
- When comparing colour intensity, compare the dot on the left of the test strips with the dot on the left of the positive control. Similarly, compare the colour intensity on the right of the strips (next to the line marked on the strips) with the colour intensity of the dot on the right of the positive control strip. Only when both dots on the test strips are as dark as or are darker than their corresponding dots on the positive control strips, the result is reported as “positive”
- If one of the dots on the test strips is lighter compared to the corresponding dot on the positive control strips, the result should be reported as “negative”
- If you are not sure i.e. borderline cases, ask for the repeat serum specimen one or two days later

Note: The strips should have a white background with only 2 dots appearing where applicable. The test should be repeated under the following conditions

- If the strips show a blue background making interpretations difficult
- If the colour intensity of the negative control is similar to that of the positive control
- If the colour intensity of the test serum is high but not equal to the positive control (request for a second serum specimen at least 2 days later so that a higher serum titre would be available for detection).

TyphiDot Results and Clinical Interpretation

Result	Clinical interpretation
IgM positive only	Acute enteric fever
IgM and IgG positive	Acute enteric fever (in the middle stage of infection)
IgG positive and IgM negative	Implications for the presence of IgG antibodies include previous infection (in which case current fever may not be due to typhoid), or relapse or re-infection, therefore it is important that interpretation be made together with clinical symptoms
IgM and IgG negative	Probably not enteric fever

ELISA FOR DENGUE & CHIKUNGUNYA

Collection, storage and transportation of samples

1. Collection of samples for isolation of Dengue virus (transportation to referral laboratory)

Specimens that may be suitable for virus isolation include:

- Acute phase serum
- Plasma
- Washed buffy coat from the patient
- Autopsy tissues especially liver, spleen, lymph nodes & thymus
- Mosquitoes collected in nature

These samples should be immediately transported (within 48 hours) to referral laboratory in an ice box.

2. Sample for serology

- Collect a blood sample as soon as possible after the onset of illness. (This is called the acute serum, S1)
- Collect a second blood sample 10-14 days after first sample or in the event of a fatality, at the time of death (convalescent serum, S2)
- Serological diagnoses are predicted on the identification of changes in antibody levels over time
- Serial (paired) specimens are required to confirm or refute a diagnosis of acute flavivirus or dengue infection.

Blood collection in tubes or vials and transportation

- Aseptically collect 2-10 ml of venous blood. The name of the patient, identification number and date of collection must be indicated on the label of the tube.
- Transport specimens to the laboratory in an ice box as soon as possible. If there is more than a 24-hour delay before specimens can be submitted to the laboratory, the serum should be separated from the RBCs and stored frozen.

Serological tests

Serological tests carried out for diagnosis of dengue are:

- Dengue IgM ELISA test
- Dengue IgM & IgG Rapid Strip Test

DENGUE IGM ELISA TEST

The kits are commercially available and the manufacturer's instructions as per kit insert should be strictly adhered to. The following protocol refers to the Dengue kit from the National Institute of Virology.

Principle

IgM antibodies in the patient's serum are captured by anti-human IgM (μ chain specific) that are coated on to the solid surface of the wells. Then the dengue antigen is added which binds to captured IgM, if the antigen and IgM are homologous. Unbound antigen is removed in the washing step and subsequently, biotinylated flavivirus cross-reactive monoclonal antibody (Hx-B) is added followed by Avidin-HRP. Addition of substrate / chromogen followed by development of colour and the reaction is stopped by 1N H₂SO₄. The optical density readings are taken at 450nm and are directly proportional to the amount of Dengue specific IgM antibodies in the sample.

Sample

- Serum (from clotted blood) - stable for weeks at 2-8°C

Equipment

- ELISA reader & washer
- Dispensing pipettes

Reagents provided

- Anti-human IgM coated test strips
- Dengue antigen
- Conjugated flavivirus cross reactive monoclonal antibody (MAb)
- Controls: (Positive /Negative)
- Conjugate. Protect the solution from direct exposure to light.
- Wash buffer concentrate
- Sample dilution buffer
- Substrate
- Stop solution (1N H₂SO₄)

Procedure

- Dilute serum with sample dilution buffer as per kit recommendation
- Wash the coated wells thrice with wash buffer
- Transfer required amount of diluted sample to the appropriate wells
- Add given positive control & negative control to the respective wells
- Keep the plate in a humidifying box, and incubate the plate at required temperature for the given time

- Wash the plate for the given no. of times with wash buffer - tap the plate after last wash on a tissue paper
- Add required amount of antigen to each well
- Repeat step 5 & 6
- Add provided MAb to each well
- Repeat step 5 & 6
- Add given amount of conjugate to each well
- Repeat step 5 & 6
- Add given amount of diluted substrate to each well (refer reagents provided)
- Incubate the plate in dark at 25°C; wait for the development of colour which normally takes 10 minutes
- Stop the reaction with given amount of 1N H₂SO₄
- Measure the absorbance at the given nm within the given time using ELISA reader

Quality Control

- Negative & positive controls are for the validation of the kit.
- Expected values for the positive & negative control & interpretation of the results will be as per the product insert.

Please amend the assay procedure as per the literature supplied in the kit.

The procedure of the Chikungunya ELISA should also be similarly adapted from the kit literature.

ELISA FOR HEPATITIS A & E

A number of commercial immunoassays for HAV specific IgM & HEV specific IgM are available. *Perform the test according to manufacturer's instructions.*

Collection Transport and Storage of specimens

- Either human serum or plasma may be used
- If the assay is performed within 48 hrs of sample collection, the sample should be kept at 2-8°C, otherwise they should be aliquoted and stored deep-frozen

IgM ELISA - HAV

Principle

The assay is an antibody-capture; non-competitive test based on the use of polystyrene microwells coated with mouse monoclonal antibody to human IgM and an enzyme tracer containing horseradish peroxidase-labelled mouse monoclonal antibody to HAV. In the assay procedure, patient sample and controls are incubated in antibody-coated microwells. IgM antibodies

present in a sample or control bind to the antibody. Excess sample is removed by a washing step and a solution of hepatitis A virus and enzyme tracer are then added to microwells and allowed to incubate. The presence of IgM anti-HAV enables the HAV and the enzyme tracer to bind to the solid phase. Excess enzyme tracer and HAV removed by washing step, and a chromogen/substrate solution is added to microwells and allowed to incubate. If sample contains IgM anti-HAV, the bound enzyme (horse radish peroxidase) chemically reduces the substrate peroxide which concurrently oxidizes to chromogen tetramethyl benzidine (TMB) to a blue colour (650 nm). The blue colour turns to yellow (450 nm) after addition of stop solution. If the sample does not contain IgM anti-HAV, the microwells will be colourless after the substrate solution is added and will remain colourless after stop solution is added. Colour intensity, which is measured spectrophotometrically by the ELISA reader, is indicative of the presence of IgM anti HAV. Absorbance value readings for patient sample are compared to cut-off value determined from the mean of calibrators.

Equipment

- Test tubes
- ELISA kits
- ELISA Reader & Washer
- Dispensing Pipettes

Reagents

- Coated strips: Wells are coated with antibody (mouse monoclonal) to human IgM
- Enzyme tracer (conjugate): before use, dilute the solution with diluent as advised
- Negative control
- Positive control
- Sample diluent
- Wash buffer
- Chromogen / substrate
- Stop solution

Procedure

- Bring all reagents to room temperature
- Specimen dilution: All samples should be diluted with sample diluent as per kit instructions, before assaying
- Fit the strip holder with required number of microELISA strips. Remove the strip sealer
- Pipette recommended volume of samples (diluted) and controls into assigned wells.
- Incubate for at 37°C as per kit instructions
- Wash repeatedly with wash buffer (5 times)
- Pipette the recommended volume of conjugate into all wells, except the blank well

- Incubate at 37°C for the recommended duration
- Wash repeatedly with wash buffer (5 times)
- Pipette recommended volume of chromogen/substrate into all wells
- Incubate for the recommended duration at RT (upto 25°C) in the dark
- Pipette recommended volume of stop solution into all wells
- Read absorbance values with an ELISA reader at recommended wavelengths.

Internal quality control

Positive & negative controls are run in each assay to validate the test

Validation

- The absorbance value for blank, negative control(s) and positive control(s) should be within the limits as per kit recommendations
- The difference between the PC and NC absorbance value must be within the recommended range
- If the validation criteria are not met, the run is invalid and must be repeated

Interpretation of the result

- Calculate the cut-off value as per kit instructions
- The unknown sample with absorbance value above the cut-off value should be considered reactive for IgM HAV
- Samples with absorbance below the cut-off should be considered as non reactive

Safety precautions - Standard precautions

Please amend the procedure as per the assay procedure given in the literature supplied in the kit.

The procedure of the IgM HEV ELISA should also be similarly adapted from the kit literature.

RAPID TEST FOR LEPTOSPIROSIS

Leptospira agglutination test

The LeptoTek Dri-Dot is used for rapid screening for leptospirosis. The assay is aimed at the detection of Leptospira-specific antibodies in human sera. The following assay refers to the procedure of the LeptoTek Dri-Dot test.

Principle

Coloured latex particles – dried and activated with a broadly reactive Leptospira antigen are provided on an agglutination card. The assay is based on the binding of Leptospira specific antibodies, present in the serum sample to the Leptospira antigen

causing a fine granular agglutination that tends to settle at the edge of the droplet (positive result). The broadly reactive antigen allows the detection of *Leptospira* infections caused by a wide range of strains of different serovars. When no specific antibodies are present the blue suspension will remain homogeneous (negative result).

Sample

Fresh serum samples are preferred. But samples can be stored for a longer period at -20°C. Dri-Dot cards can be stored at 2-4°C in a dry place protected from direct exposure to the sun.

Requirements

- 10µl micropipette
- Disposable pipette tips

Procedure

- Take out a Dri-Dot card from the packaging and place the card on a bench top with the blue dot facing upwards
- Spot 10 µl serum next to the blue dot and within the area marked by the black circle
- Break off a spatula and hold with the flat surface facing downwards
- Hold the spatula with thumb and forefinger close to the flat end of the spatula
- Suspend the blue dot in the serum with a quick circular motion while pressing the flat end of the spatula on to the dot
- Do not spread the suspension outside the area marked by the black circle
- Proceed with the next step when the blue dot is fully suspended and a homogeneous suspension is obtained
 1. Suspending of the blue dot with the spatula should only take a few seconds
 2. The suspension should not be spread outside the area marked by the black circle, as spreading over a large surface will hamper the mixing procedure
- Keeping the card near horizontal slowly rotate the card swirling the liquid in a circular motion in order to further mix latex and serum sample and to induce agglutination
- Read result within 30 seconds after start of mixing
 1. Always read the result within 30 seconds, otherwise false results may occur
 2. As with any diagnostic assay, results from the Lepto Tek Dri Dot should be interpreted with consideration of the clinical, epidemiological and other laboratory findings

Reading of results

- Aggregation of the latex particles of the test dot reveals agglutination by *Leptospira* specific antibodies present in the serum samples
- The degree of agglutination depends on the amount of specific antibodies in the sample which is relative to the stage of the disease as well as on other factors
- The sensitivity of the assay is highest for samples collected 10 to 30 days after the onset of the disease

- Strong agglutination visible within 30 seconds is highly consistent with current or recent leptospirosis
- As antibodies reach detectable levels about one week after the onset of the disease, a serum sample collected very early in the disease may fail to cause agglutination in this assay
- If the assay performed on a serum sample collected in the early phase of the disease is negative, and suspicion of leptospirosis remains
- It is advised to examine a second sample collected some days after the first sample
- As with any diagnostic assay, results from the Lepto Tek Dri Dot should be interpreted with consideration of the clinical, epidemiological and other laboratory findings

Leptospira ELISA Test

ELISA test is an enzyme immunoassay for the detection of antibodies to *Leptospira biflexa* for the serological confirmation of infections in serum, and plasma. The kits are commercially available and manufacturer's instructions should be strictly followed.

DIRECT DETECTION OF MENINGOCOCCI IN CSF (GRAM STAINING & LATEX AGGLUTINATION)

Samples that can be collected include:

- Cerebrospinal fluid
- Blood
- Petechial fluid (in cases with meningococcaemia)

Collection, storage and transportation of specimen

Cerebrospinal fluid

- Collect CSF before commencement of antimicrobial therapy, but treatment must not be delayed pending lumbar puncture
- The collection of CSF is an invasive procedure and should be performed only by experienced personnel under aseptic conditions
- Send the CSF to laboratory as early as possible preferably within an hour
- Do not expose the CSF to sunlight or extreme heat or cold
- Never pipette CSF by mouth
- Never freeze or refrigerate the CSF in suspected pyogenic meningitis
- It is preferable to make a smear for direct demonstration of organisms from CSF at the time of collection itself
- Culture as rapidly as possible; in case of delay incubate at 37°C
- CSF is a precious specimen since a repeat is extremely difficult to obtain

Blood

- Blood cultures from young children should have 1-2 ml of blood in 20 ml of broth (1:10 to 1:20 dilution)
- Blood cultures from adults should have 5-10 ml of blood in 50 ml of broth (1:5 to 1:10 dilution)
- Immediately transport the inoculated media to the laboratory
- All blood-culture bottles should reach the laboratory within 12 to 18 hours for subculture and should be protected from temperature extremes (<18°C & >37°C)
- Extended culture is preferred and the inoculated media should not be discarded before seven days of incubation

Petechial Fluid

- Petechial lesions, if present may be gently irrigated by injecting 0.2 ml of sterile saline solution using a small syringe with a fine needle and the fluid re-collected for making smear and doing culture examination

CSF examination

Macroscopic examination - Gross examination should be recorded for all samples

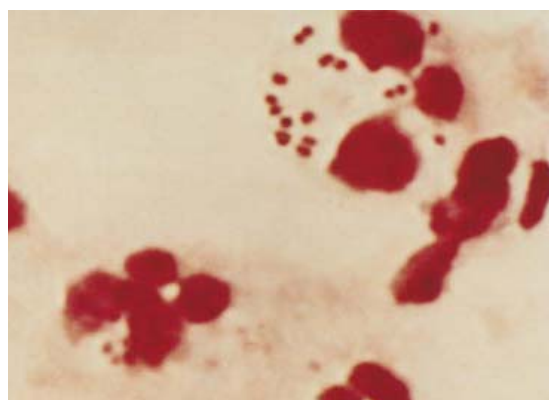
- Colour
- Presence of turbidity, deposit or clot.

Microscopic Examination

- Wet mount: Count the number of polymorphs, lymphocytes and RBC's per cubic ml of CSF using a white cell counting chamber

Gram stain

Make a smear using CSF sediment. *N. meningitidis* may occur intra- (or extra)-cellularly in the polymorphonuclear leukocytes and will appear as Gram-negative, coffee-bean shaped diplococci



Gram stain of CSF — N. meningitidis seen as intra-cellular, Gram-negative diplococci

Biochemical and cytological examination

- Sugar and protein levels should be estimated and cytochemical examination done for the presence and number of polymorphonuclear cells

Latex agglutination tests

Several commercial tests based on latex agglutination principle are available. Most of the available commercial tests are designed to provide diagnosis for meningitis caused by:

- *N. meningitides*
- *H. influenzae* type B
- *S. pneumoniae*
- *E. coli* K1
- Group B streptococci

Performance of the test

- Heat the supernatant of the CSF in a boiling water bath at 80-100°C for 5 minutes
- Shake the latex suspension gently until homogenous
- Place one drop of each latex suspension on a rinsed glass slide or a disposable card
- Add 30 - 50µl of CSF to each suspension
- Mix with a stirring stick (use one stick per specimen)
- Rotate by hand for 2-10 minutes
- Mechanical rotation at 100 rotations per minutes if available is recommended
- Read the results within 2 minutes under bright light

Reading & Interpretation

- Negative reaction - the suspension remains homogenous and slightly milky in appearance
- Positive reaction - visible clumping of the latex particles within 2 minutes
- Agglutination with one of the latex reagents indicates the presence of the corresponding antigen in the CSF sample

Controls

Periodically check

- That none of the latex reagents agglutinate in the presence of normal saline
- That each of the latex reagents do agglutinate with respective positive controls

General recommendations and instructions for detection of soluble bacterial antigens

- Store the reagents at 2-8°C; latex suspensions should not be frozen
- Test the supernatant of the centrifuged CSF sample as soon as possible
- In case of delay, refrigerate the sample at 2-8°C (up to several hours) or at -20°C (for longer periods)
- Follow the kit manufacturer's instructions precisely when using these tests

ELISA FOR MEASLES

- This is a test to detect IgM antibody against measles virus in human serum. Samples negative for measles may be screened for Rubella IgM antibodies.

Principle

The ELISA method is based upon the reaction of antibodies in the sample tested with the antigen adsorbed on the polystyrene surface. An enzyme-labelled anti-human globulin binds the antigen-antibody complex in a second step. After a washing step, bound conjugate is developed with the aid of a substrate solution to render a blue coloured soluble product which turns into yellow after adding the acid stopping solution.

Perform the test according to manufacturer's instructions.

All reagents, except washing solution are ready to use. Serum dilution solution and conjugate are coloured.

Kit contents

- ELISA plate - microwell plate coated with measles antigen, strain Edmonston
- Serum diluent
- Controls – IgM positive control serum, IgM cut off control serum & IgM negative control serum
- IgM conjugate - anti-human IgM peroxidase conjugate
- TMB (tetramethylbenzidine) substrate solution
- Stop reagent - 0.5 M sulphuric acid
- Wash buffer - concentrated solution
- Sealing sheets

Materials required

- Micropipettes (5 and 100 µl) & multi-channel micropipette (100 µl)
- Incubator / waterbath
- ELISA reader with a 450 nm measuring filter and a 620 nm reference filter
- Distilled water

Storage requirements

Store at 2-8°C. Do not use the kit reagents beyond the expiration date.

Stability and handling of reagents

- Handle reagents with aseptic precautions to avoid microbial contamination.
- Do not let the plate dry between washing and reagent addition.
- Substrate solution is light sensitive - avoid light exposure and discard if blue colour develops during storage.
- Substrate solution should not contact oxidisers such as bleach solution or metals.

Precautions

- Do not mix components from different kits or manufacturers.

- Use clean, disposable pipette tips for every assay step; never pipette by mouth.
- All the material provided in the kit should be handled and disposed as potentially infectious.
- Substrate solution may be irritant to skin & mucous membranes - in case of contact, rinse thoroughly with water and seek medical attention.

Specimen collection and handling

- Blood should be collected aseptically using venepuncture.
- Serum samples are to be refrigerated (2-8°C) upon collection or frozen (-20°C) if the test cannot be performed within 7 days.
- Samples should not be repeatedly frozen and thawed.
- Do not use haemolysed, hyperlipaemic, or contaminated sera.
- Do not use plasma.

Assay Procedure

- Set incubator/water bath to 37°C.
- Bring all reagents to room temperature, without removing the plate from the bag
- Shake all components; remove the plate from the package.
- Determine the number of wells to be used, counting four wells for the controls - 2 for the cut off, and one each for positive and negative controls (Wells not required should be returned to the pouch, and sealed).
- Add recommended amount of IgG sorbent into all wells followed by recommended amount of serum diluent to all wells.
- Cover with a sealing sheet and incubate at 37°C for the recommended period.
- Remove the seal, aspirate liquid from all wells and wash 5 times with 0.3 ml of washing solution per well; drain off any remaining liquid.
- Add recommended amount of IgM conjugate solution into each well.
- Cover with a sealing sheet and incubate at 37°C for the recommended period.
- Remove the seal, aspirate liquid from all wells and wash 5 times with 0.3 ml of washing solution per well; drain off any remaining liquid.
- Add recommended amount of substrate solution into each well.
- Cover with a sealing sheet and incubate at room temperature for 20 minutes protected from light.
- Remove the seal and add recommended amount of stopping solution into all wells; take readings using an ELISA reader, within 1 hour of stopping the reaction.

Validation protocol

- Positive, negative and cut off controls must be run with each test run.
- It allows validation of the assay and the kit.
- Optical densities must fall in the recommended ranges; otherwise the test is invalid and must be repeated.

Interpretation of results

- Interpret the sample ODs as per the kit instructions.
- Retest samples with equivocal results or obtain a new sample for confirmation.
- Samples negative for measles may be screened for Rubella IgM antibodies.

Chapter 6

ANTIMICROBIAL SUSCEPTIBILITY TESTING

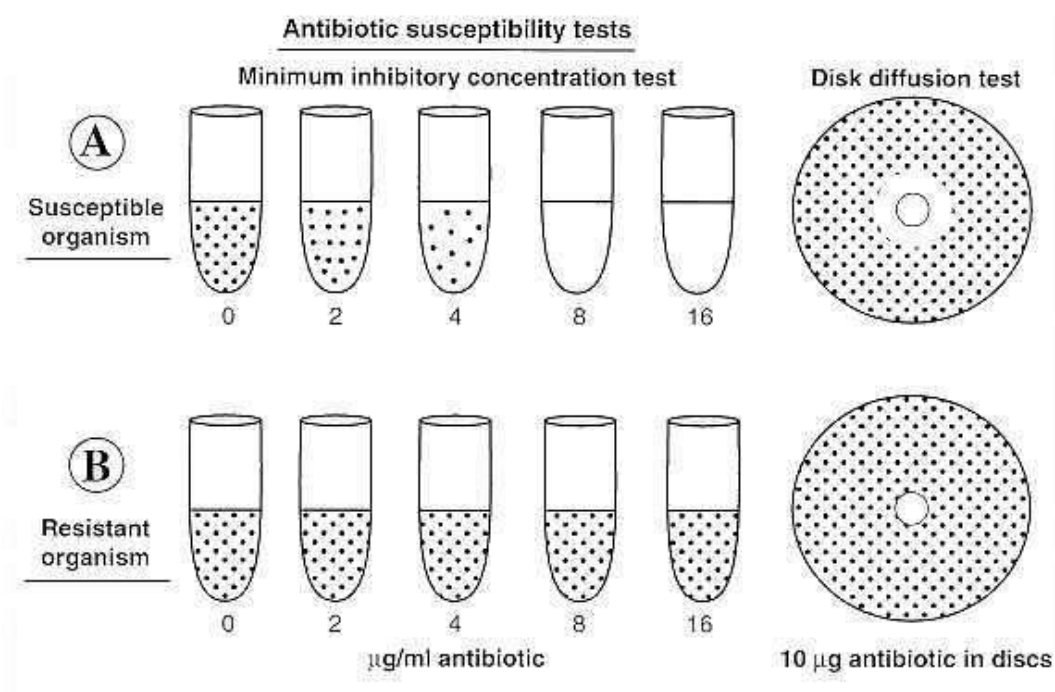
Antimicrobial susceptibility tests measure the ability of an antibiotic or other antimicrobial agent to inhibit bacterial growth *in vitro*. This ability may be estimated by either the dilution method or the diffusion method.

Dilution method

The dilution method is used for quantitative estimation of antibiotic activity. Dilutions of the antibiotic may be incorporated into broth or agar medium, which is then inoculated with the test organism. The lowest concentration that prevents growth after overnight incubation is known as the minimum inhibitory concentration (MIC) of the agent. This MIC value is then compared with known concentrations of the drug obtainable in the serum and in other body fluids to assess the likely clinical response.

Diffusion method

This is the routinely used method in most diagnostic laboratories. Paper discs, impregnated with the antibiotic, are placed on agar medium uniformly seeded with the test organism. A concentration gradient of the antibiotic forms by diffusion from the disc and the growth of the test organisms is inhibited at a distance from the disc that is related, among other factors, to the susceptibility of the organism.



Clinical definition of terms “resistant” and “susceptible”

The result of the susceptibility test, as reported to the clinician, is the classification of the microorganism in one of two or more categories of susceptibility. The simplest system comprises only two categories: susceptible and resistant. This classification, although offering many advantages for statistical and epidemiological purposes, is too inflexible for the clinician to use. Therefore, a three-category classification is often adopted. The Kirby-Bauer method and its modifications recognize three categories of susceptibility and it is important that both the clinician and the laboratory worker understand the exact definitions and the clinical significance of these categories.

- *Susceptible*: An organism is called “susceptible” to a drug when the infection caused by it is likely to respond to treatment with this drug at the recommended dosage.
- *Intermediate*: This covers two situations. It is applicable to strains that are “moderately susceptible” to an antibiotic that can be used for treatment at a higher dosage because of its low toxicity or because the antibiotic is concentrated in the focus of infection (e.g. urine). The classification also applies to strains that show “intermediate susceptibility” to a more toxic antibiotic that cannot be used at a higher dosage. In this situation, the intermediate category serves as a buffer zone between susceptible and resistant. As most clinicians are not familiar with the subtle, although clinically important, distinction between intermediate and moderate susceptibility, many laboratories use the designation “intermediate” for reporting purposes.
- *Resistant*: This term implies that the organism is expected not to respond to a given drug, irrespective of the dosage and of the location of the infection

For testing the response of staphylococci to benzylpenicillin, only the categories “susceptible” and “resistant” (corresponding to the production of β -lactamase) are recognized.

The ultimate decision to use a particular antibiotic, and the dosage to be given, will depend not only on the results of the susceptibility tests, but also on their interpretation by the physician. Other factors, such as pathogenic significance of the microorganism, side-effects and pharmacokinetic properties of the drug, its diffusion in different body sites, and the immune status of the host, will also have to be considered.

Indication for routine susceptibility tests

A susceptibility test may be performed in the clinical laboratory for two main purposes:

- Guide the clinician in selecting the best antimicrobial agent for an individual patient
- Accumulate epidemiological information on the resistance of microorganisms of public health importance within the community

Susceptibility tests as a guide for treatment

Susceptibility tests should never be performed on contaminants or commensals belonging to the normal flora, or on other organisms that have no causal relationship to the infectious process. For example, the presence of *Escherichia coli* in the urine in less than significant numbers is not to be regarded as causing infection, and it would be useless and even misleading to perform an antimicrobial susceptibility.

Susceptibility tests should be carried out only on *pure cultures* of organisms considered to be causing the infectious process. The organisms should also be identified, as not every microorganism isolated from a patient with an infection requires antimicrobial susceptibility testing to be done.

Susceptibility tests as an epidemiological tool

Routine susceptibility tests on major pathogens (*S. typhi*, *Shigella* sp) are useful as part of a comprehensive programme of surveillance of enteric infections. They are essential for informing the physician of the emergence of resistant strains (chloramphenicol-resistant *S. typhi*, co-trimoxazole-resistant and ampicillin-resistant *Shigella* sp) and of the need to modify standard treatment schemes.

Once isolated colonies are available from an organism that has been identified as a potential pathogen, it is necessary to proceed as follows to perform the susceptibility test.

1. Select colonies
2. Prepare inoculum suspension
3. Standardize inoculum suspension
4. Inoculate plate
5. Add antimicrobial disks
6. Incubate plate
7. Measure zone inhibition diameters
8. Interpret results

Kirby-Bauer method

Reagents

- Mueller-Hinton agar (MHA)
 - MHA should be prepared from a dehydrated base according to the manufacturer's recommendations. The medium should be such that control zone sizes within the published limits are produced. It is important not to overheat the medium.
 - Cool the medium to 45-50°C and pour into the plates. Allow to set on a level surface, to a depth of approximately 4 mm (a 9-cm plate requires approximately 25 ml of medium). When the agar has solidified, dry the plates for immediate use for 10-30 minutes at 35°C, by placing them in the upright position in the incubator with the lids tilted.
 - Any unused plates may be stored in a plastic bag, which should be sealed and placed in the refrigerator. Plates stored in this way will keep for 2 weeks.

- *Quality control* - To ensure that the zone diameters are sufficiently reliable for testing susceptibility to sulfonamides and co-trimoxazole, the Mueller-Hinton agar must have low concentrations of the inhibitors thymidine and thymine. Each new lot of Mueller-Hinton agar should therefore be tested with a control strain of *Enterococcus faecalis* (ATCC 29212 or 33186) and a disc of co-trimoxazole. A satisfactory lot of medium will give a distinct inhibition zone of 20 mm or more that is essentially free of hazy growth or fine colonies.
- Antibiotic discs

Any commercially available discs with the proper diameter and potency can be used. Stocks of antibiotic discs should preferably be kept at -20°C; the freezer compartment of a regular refrigerator is convenient. A small working supply of discs can be kept in the refrigerator for up to 1 month. On removal from the refrigerator, the containers should be left at room temperature for about 1 hour to allow the temperature to equilibrate. This procedure reduces the amount of condensation that occurs when warm air reaches the cold container. Do not use discs beyond their expiry dates.
- Turbidity standard

Prepare the turbidity standard by pouring 0.6 ml of a 1% (10 g/litre) solution of barium chloride dehydrate into a 100-ml graduated cylinder, and filling to 100 ml with 1% (10 ml/litre) sulphuric acid. The turbidity standard solution should be placed in a tube identical to the one used for making the suspension. It can be stored in the dark at room temperature for 6 months, provided it is sealed to prevent evaporation.
- Swabs

A supply of cotton wool swabs on wooden applicator sticks should be prepared. They can be sterilized in tins, culture tubes, or on paper, preferably by dry heat.

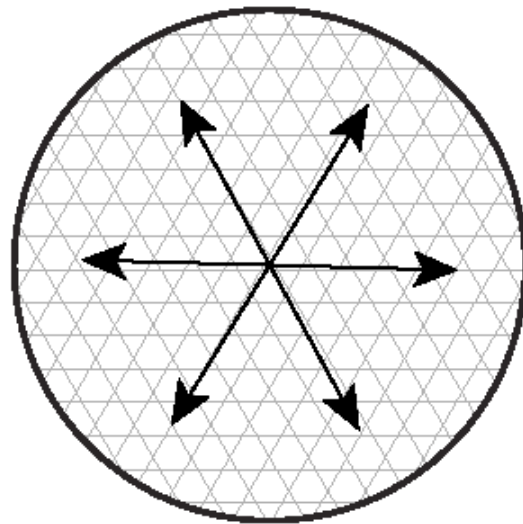
Procedure

- To prepare the inoculum from the primary culture plate, touch with a loop the tops of each of 3-5 colonies, of similar appearance, of the organism to be tested.
- Transfer this growth to a tube of saline.
- When the inoculum has to be made from a pure culture, a loopful of the confluent growth is similarly suspended in saline.
- Compare the tube with the turbidity standard and adjust the density of the test suspension to that of the standard by adding more bacteria or more sterile saline.
- Proper adjustment of the turbidity of the inoculum is essential to ensure that the resulting lawn of growth is confluent or almost confluent.
- Inoculate the plates by dipping a sterile swab into the inoculum. Remove excess inoculum by pressing and rotating the swab firmly against the side of the tube above the level of the liquid.



Standardizing the inoculum

- Streak the swab all over the surface of the medium three times, rotating the plate through an angle of 60 °C after each application. Finally, pass the swab round the edge of the agar surface. Leave the inoculum to dry for a few minutes at room temperature with the lid closed.



- The antibiotic discs may be placed on the inoculated plates using a pair of sterile forceps. It is convenient to use a template to place the discs uniformly.

- A sterile needle tip may also be used to place the antibiotic discs on the plate.

- Alternatively, an antibiotic disc dispenser can be used to apply the discs to the inoculated plate.

- A maximum of seven discs can be placed on a 9-10 cm plate. Six discs may be spaced evenly, approximately 15 mm from the edge of the plate, and one disc placed in the centre of the plate. Each disc should be gently pressed down to ensure even contact with the medium. Once discs have touched the medium, do not relocate them.

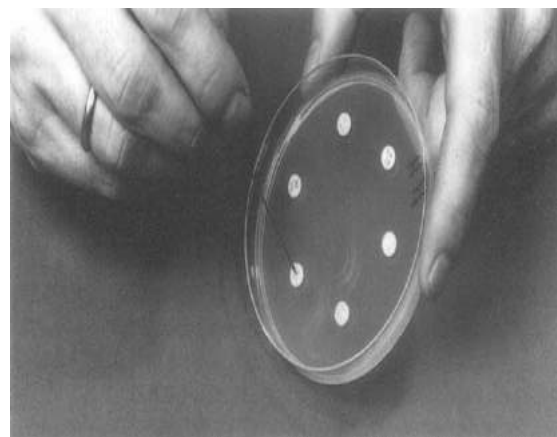


Inoculation of plate

- The plates should be placed in an incubator at 35°C within 30 minutes of preparation. Temperatures above 35°C invalidate results for oxacillin/methicillin.

- Do not incubate in an atmosphere of carbon dioxide.

- After overnight incubation, the diameter of each zone (including the diameter of the disc) should be measured and recorded in mm. The results should then be interpreted according to the critical diameters.



Ensure contact of the discs with the medium

- The measurements can be made with a ruler on the under-surface of the plate without opening the lid.

- If the medium is opaque, the zone can be measured by means of a pair of calipers. A template may be used to assess the final result of the susceptibility tests.

Table 6.1 – Interpretative chart of zone inhibition sizes

Antibiotic or chemotherapeutic agent	Diameter of zone of inhibition (mm)			
	Disc potency	Resistant	Intermediate / moderately susceptible	Susceptible
Amikacin	30 µg	≤ 14	15-16	≥ 17
Ampicillin when testing:				
- <i>Enterobacteriaceae</i>	10 µg	≤ 13	14-16	≥ 17
- <i>Enterococcus faecalis</i>	10 µg	≤ 16	-	≥ 17
Benzylpenicillin when testing staphylococci	10 IU	≤ 28	-	≥ 29
Ceftriaxone	30 µg	≤ 13	14-20	≥ 21
Cefuroxime sodium	30 µg	≤ 14	15-17	≥ 18
Cefalothin	30 µg	≤ 14	15-17	≥ 18
Chloramphenicol	30 µg	≤ 12	13-17	≥ 18
Clindamycin	2 µg	≤ 14	15-20	≥ 21
Co-trimoxazole	25 µg	≤ 10	11-15	≥ 16
Erythromycin	15 µg	≤ 13	14-22	≥ 23
Gentamicin	10 µg	≤ 12	13-14	≥ 15
Nalidixic acid	30 µg	≤ 13	14-18	≥ 19
Nitrofurantoin	300 µg	≤ 14	15-16	≥ 17
Oxacillin when testing:				
- <i>Staphylococci</i>	1 µg	≤ 10	11-12	≥ 13
- <i>Pneumococci</i>	1 µg	≤ 19	-	≥ 20
Tetracycline	30 µg	≤ 14	15-18	≥ 19

Technical factors influencing the size of the zone in the disc diffusion method

- *Inoculum density*
If the inoculum is too light, the inhibition zones will be larger although the sensitivity of the organism is unchanged. Relatively resistant strains may then be reported as susceptible. Conversely, if the inoculum is too heavy, the zone size will be reduced and susceptible strains may be reported as resistant. Usually optimum results are obtained with an inoculum size that produces near confluent growth.
- *Timing of disc application*
If the plates, after being seeded with the test strain, are left at room temperature for periods longer than the standard time, multiplication of the inoculum may take place before the discs are applied. This causes a reduction in the zone diameter and may result in a susceptible strain being reported as resistant.
- *Temperature and time of incubation*
Susceptibility tests are normally incubated at 35°C for optimal growth. If the temperature is lowered, the time required for effective growth is extended and larger zones result. At higher temperature the entire culture appears to be susceptible. Mostly an incubation period of between 16 to 18 hours is adopted (overnight incubation).
- *Size of plate, depth of agar medium, and spacing of the antibiotic discs*
Susceptibility tests are usually carried out with 9-10 cm plates and no more than 6 or 7 antibiotic discs on each plate. A very thin media may lead to formation of excessively large inhibition zones and vice versa. Minor changes in depth of agar

layer have negligible effect. Proper spacing of the discs is essential to avoid overlapping of inhibition zones or deformation near edge of the plates.

▫ *Composition of the medium*

The medium influences the size of the zone by its effect on the rate of growth of the organism, the rate of diffusion of the antibiotic and the activity of the agent.

Chapter 7

BIOSAFETY MEASURES IN LABORATORY

“Laboratory bio-safety” is the term used to describe the practices that are implemented to prevent unintentional exposure to pathogens and toxins, or their accidental release. Bio-safety protection is to protect laboratory workers, clinical samples and the environment. As no laboratory has complete control over the specimens it receives, standard precautions should always be adopted and practiced.

Entry / access to laboratory area

- Entry to laboratory working area should be restricted only for authorized persons.
- Doors of the laboratory should be kept closed.
- Children should not be allowed to enter laboratory working areas.

Laboratory Design and Facilities

- Enough space should be available
- Smooth easily cleanable walls, ceiling and floors impermeable to liquids and resistant to chemicals and disinfectants, should be preferred
- Bench tops should be impervious to water and resistant to disinfectants
- Ample illumination should be available for laboratory procedures
- Storage space must be adequate to hold supplies for immediate use and to prevent overcrowding on bench tops
- Regular, water supply should be available
- Wash basins with running water, if possible, should be provided in the laboratory room, preferably near the exit door
- Suitably equipped first aid box should be available in the laboratory
- Rodents and insects control procedure in the laboratory should be in place

Standard precautions

These combine the major features of Universal Precautions and Body Substance Isolation and are based on the principle that all blood, body fluids, secretions, excretions except sweat, non-intact skin, and mucous membranes may contain transmissible infectious agents.

Standard Precautions include a group of infection prevention practices that apply to all patients, regardless of suspected or confirmed infection status, in any setting in which healthcare is delivered.

These include: hand hygiene; use of gloves, gown, mask, eye protection, or face shield, depending on the anticipated exposure; and safe injection practices. Also, equipment or items in the patient environment likely to have been contaminated with infectious body fluids must be handled in a manner to prevent transmission of

infectious agents (e.g., wear gloves for direct contact, contain heavily soiled equipment, properly clean and disinfect or sterilize reusable equipment before use on another patient).

The application of Standard Precautions during patient care is determined by the nature of the HCW-patient interaction and the extent of anticipated blood, body fluid, or pathogen exposure. For some interactions (e.g., performing venepuncture), only gloves may be needed; during other interactions (e.g., intubation), use of gloves, gown, and face shield or mask and goggles is necessary.

Standard Precautions are also intended to protect patients by ensuring that healthcare personnel do not carry infectious agents to patients on their hands or via equipment used during patient care.

Some of the important components of standard precautions are as under:

Personal Protective Equipment

1. *Gloves* can reduce the incidents of contamination of hands but cannot prevent penetrating injuries by needles and other sharp instruments. Gloves should be:

- Well fitting, disposable, vinyl
- Heavy duty general purpose rubber gloves for washing infected glassware/sharps



Use Gloves

Uses of Gloves

- Worn while collecting/handling blood specimens, blood soiled items or whenever there is a possibility of exposure to blood or body fluids containing blood
- Worn while disposing laboratory waste

When to change gloves

- The utility gloves may be decontaminated and reused but should be discarded if they are peeling, cracked, discoloured, or if they have puncture, tears, etc
- Should be removed before handling door knobs, telephones, pens, performing office work and leaving the laboratory
- Must be changed if visibly contaminated with blood/breached

2. *Laboratory gowns*

- Laboratory gowns or uniforms (preferably wrap-around gowns) should be worn when in the laboratory and should be removed before leaving
- Plastic aprons should be used while cleaning infected re-usable and during disposing wastes

3. *Facial protection*

- Simple and cheap deflector masks and protective glasses may be worn if splashing or spraying of blood/body fluids is expected

4. Occlusive bandage

- All skin defects e.g. Cuts, scratches or other breaks must be covered with water-proof dressing before handling infectious materials

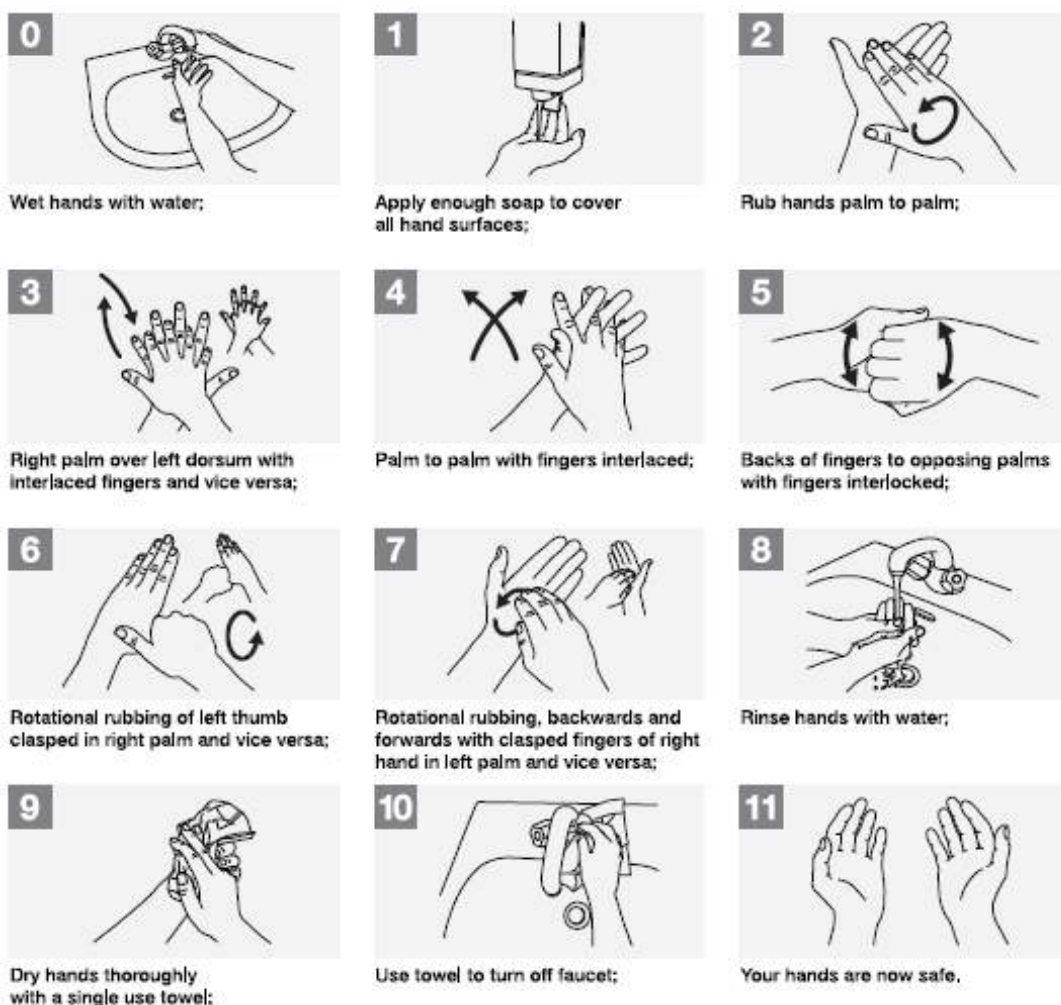
Hand washing

Hand washing is the single most important means of preventing the spread of infection.

- Hands should be washed between patient contacts and after contact with blood/body fluids, secretions, excretions and equipment s or articles contaminated by these.
- The role of hands in the transmission of infections has been well demonstrated, and can be minimized with appropriate hand hygiene.
- Hands should be washed thoroughly in running water with soap without missing any area

Washing of hands is mandatory after

- Contamination with blood / body fluids
- After removing gowns / coats and gloves
- Before eating / drinking and leaving the laboratory



Steps for good hand washing

The following facilities are required:

- Running water: large washbasins preferably with hands free controls, which require little maintenance and with anti-splash devices
- Products: dry soap or liquid antiseptic depending on the procedure. - Ideally, liquid soap dispensers should be provided to the laboratories, which should be regularly cleaned and maintained. If not feasible, soap bars after washing should be left in a dry tray to prevent contamination with some microorganisms which grow in moist conditions
- Suitable material for drying of hands; disposable towels, reusable sterile single use towels or roller towels which are suitably maintained

Gloves should not be regarded as substitute for hand washing

Safe techniques

- All procedures and manipulations of potentially infectious material should be performed in a separate area, to minimize the formation of droplets, aerosols, splashes or spills
- Mouth pipetting should be strictly prohibited. Mechanical pipetting devices should be used for pipetting of all liquids in the laboratory.
- Centrifugation should be done in tubes with safety caps



Pipetting

Safe handling of sharps

- Extreme care should be used to avoid auto-inoculation.
- All chipped or cracked glassware should be discarded in appropriate containers.
- Broken glass should be picked up with a brush and pan. Hands must never be used.
- The disposable needles should never be manipulated, bent, broken, recapped or removed from the syringes.
- The used sharps should never be passed directly from one person to another.
- Each healthcare worker should dispose of his / her own sharps.
- Used needles should be discarded in puncture-proof rigid containers (plastic or cardboard boxes) after disinfection in 0.5-1% freshly prepared sodium hypochlorite solution (common bleach) only. Do not mix with other waste. If a needle shredder is available, only the needles or the needles along with syringe nozzle may be shredded depending upon the type of the shredder
- Sharp disposable containers should be located close to the point of use.
- Sharp disposal containers should be sent for disposal when three-fourths full.



To pick broken glass, use brush & pan or pieces of cardboard



Needle shredder

Precautions for safe handling of sharps

Do's	Don'ts
Pass syringes and needles in a tray. Preferably cut it with needle cutter	Never pass syringes and needle directly to next person
Put needle and syringes in 2% hypochlorite solution if needle cutter is not available	Do not bend or break or recap used needle with hands
Remove cap of needle near the site of use	Never test the fineness of the needle's tip before use with bare or gloved hand
Pick up open needle from tray/drum with forceps	Never pick up open needle by hand
Destroy syringes by burning their tips/or if cutters not available	Never dispose it off by breaking it with hammer/stone

Safe handling of specimens

- Specimens, specially blood and body fluids should be collected in pre-sterilized screwcapped containers properly sealed to prevent spillage or leakage.
- Pre-sterilized /autoclaved / disposable syringes and needles for venepuncture or lancets / cutting needles for finger prick should be used. Auto destructive syringes if available are a good alternative.
- Cuts in hands should be properly covered with waterproof adhesive bandages.
- Disposable gloves should be worn while collecting blood / body fluids and proper asepsis should be maintained.
- If a sample shows evidence of breakage (in case not collected in the above container), leakage or soiling, it should be transferred with a gloved hand into a second sterile container. Any important information should be rewritten from the old to the new container. This should only be done if sample is highly precious otherwise it should be discarded and fresh sample to be collected.
- Do not keep samples on requisition forms.
- If the requisition slip is contaminated with blood, it should be rejected. In case of emergency, the contaminated slip may be handled using gloves.
- Hands should be thoroughly washed with soap and water before and after handling specimens.

Specimen containers

- Specimen containers may be of glass or preferably plastic.
- They should be robust and should not leak when the cap or stopper is correctly applied.
- A specimen container without cap/stopper is simply unacceptable for Sample collection.
- No material should remain on the outside of the container.
- Containers should be correctly labelled to facilitate identification.
- Do not put samples on request forms.
- Specimen request or specification forms should not be wrapped around the containers but placed in separate, preferably waterproof envelopes, whenever the specimen needs to be transported.

Transport of specimens within the facility

- To avoid accidental leakage or spillage, secondary containers, such as boxes, should be used, fitted with racks so that the specimen containers remain upright.
- The secondary containers may be of metal or plastic, should be autoclavable or resistant to the action of chemical disinfectants, and the seal should preferably have a gasket. They should be regularly decontaminated

Separation of serum: Always take care of following:

- PPE for hands, eye and mucous membrane protection should be used (Standard precautions)
- Splashes and aerosols can only be avoided or minimized by good laboratory techniques
- Blood and serum should be pipetted carefully, not poured
- Mouth-pipetting is forbidden
- After use, pipettes should be completely submerged in suitable disinfectant - they should remain in the disinfectant for the appropriate time before disposal or washing and sterilization for reuse (15 minutes).
- Discarded specimen tubes containing blood clots, etc. (with caps replaced) should be placed in suitable leak-proof containers for autoclaving and/ or incineration
- Suitable disinfectants should be available for clean- up of splashes and spillages

Safe handling of blood / body fluid spills

In case of a spill of blood / body fluid in the laboratory, the area should be flooded with a disinfectant solution e.g. freshly prepared 0.5-1% sodium hypochlorite solution and left for 10 minutes. After wearing gloves, the area should be covered with paper towels or gauze sponges to absorb the liquid followed by a thorough wash with soap and water. All contaminated materials should be disposed of as infectious waste.

Procedure to clean up spills

- Pour 1% freshly prepared sodium hypochlorite solution over spills in sufficient quantity
- Cover the spill with paper towel or absorbent material
- Leave for 10 minutes before cleaning it
- Wipe up the whole spill with fresh absorbent material using gloved hands and discard in a contaminated waste container
- Wipe the surface with soap and water
- Record the event

Laundry and linen

- Although soiled linen has been identified as a source of large numbers of certain pathogenic organisms, the risk of actual disease transmission is negligible
- Soiled linen may be handled as little as possible and with minimum agitation to prevent gross microbial contamination of the air and of persons handling the linen
- All soiled linen must be handled with gloved hands and if feasible, decontaminated in 0.5- 1% sodium hypochlorite in the laboratory before sending to the laundry
- Soiled linen after decontamination should be put in heavy plastic bags which are tied and sent to the laundry
- In the laundry, decontamination in bleach is recommended in case not done earlier. This should be followed by washing in hot water (>70°C) with detergent

General biosafety rules for laboratory workers

- Eating, drinking, smoking and application of cosmetics are prohibited in the laboratory.
- Laboratory should be kept neat, clean and free of materials not pertinent to the work.
- Sandals and open shoes do not afford proper foot protection and should be avoided.
- As far as possible contact lenses should not be worn, instead wear spectacles.
- Laboratory and work tables should be scrupulously cleaned with liquid detergents and disinfectants. Laboratory work surface should be decontaminated twice daily, once before beginning work and once after completion of day's activity. It should also be cleaned immediately after spill of viable material with disinfectant.
- Centrifuge safety caps should be used whenever handling hazardous specimens and when it is likely to produce aerosols or infectious droplets.
- Blood and other specimen containers should be labelled with a warning sign. The outside of the specimen container should be cleaned with sodium hypochlorite solution in case of visible contamination.
- Gloves should be worn while dealing with blood specimens, blood-soiled items, body fluids, excretions, secretions, surface materials and objects exposed to them.
- Hands should be washed immediately after contact with blood and before leaving the laboratory.
- Hands should always be washed before wearing and after removing gloves.
- Gowns / laboratory coats must be worn while working with potentially infective materials and removed before leaving the laboratory.
- *Recapping or bending of needles is strictly prohibited.* Needles should be destroyed with needle destroyer and remaining hub should be put in puncture proof container.
- Syringes should be put in freshly prepared 1% sodium hypochlorite solution.
- Paper work should not be done on potentially contaminated surface.
- All potentially contaminated materials and wastes from the laboratory should be disposed after decontamination preferably by autoclaving / incineration.

Biosafety management

- Designate one person responsible for bio-safety activities, e.g. DMS / Microbiologist / MO CHC / PHC or senior laboratory technician.
- Appropriate medical evaluation, surveillance and treatment should be provided for all personnel in case of need, and adequate medical records should be maintained.
- Immunization against diseases which are feasible must be given according to the schedule, especially against Hepatitis B, etc.
- Staff should receive regular training in laboratory bio-safety and should be updated at regular interval. A copy of the Bio-safety manual should be available in the laboratory at all times.

Training

- Human error and poor techniques are important in non protection of laboratory workers.
- Continuous in-service training in safety measures is essential for health care workers to minimize human errors and improve laboratory techniques. An effective safety program begins with the laboratory in-charge, who should ensure that safe laboratory practices and procedures are being followed.
- Staff training should include safe methods adopted for commonly used laboratory procedures like:
 - Inhalation risks – using loops, streaking agar plate, pipetting, smear preparation, opening culture stocks, centrifugation, taking blood/serum samples etc.
 - Ingestion risks – handling specimens, smears, cultures.
 - Inoculation risks – accidental needle stick injuries.
 - Handling blood and other infectious agents.
 - Decontamination and disposal of infectious material.

Health and medical check up

The microbiologist / pathologist at the district public health laboratory could help in ensuring that there is regular health check up of laboratory personnel. The objective of such check up is to monitor for occupationally acquired diseases.

- Provision of immunization: It is recommended that all laboratory persons receive protective immunization against the disease they are dealing with especially **Hepatitis B** which plays a key role in prevention of transmission of HBV from patient to health care worker. Therefore it is important that all the HCW's including the laboratory workers should be immunized with 3 doses of hepatitis B as per approved schedule (0, 1 and 6 months interval). Health care worker should receive booster dose after 5-6 years interval.
- Regular health check-up of the staff for diseases including collection of samples where ever indicated. The record for the same should be maintained.

Good Microbiological Techniques - Safe laboratory procedures

Human error, poor laboratory techniques and misuse of equipment cause the majority of laboratory injuries and work-related infections. This chapter provides a

short note on technical methods that are designed to avoid or minimize the most commonly reported problems of this nature.

Receipt of specimens

- Laboratories that receive large numbers of specimens should have a designated room or area for this purpose. It will be preferred to have computerized system for record maintenance.

Opening packages

- Personnel *who receive and unpack specimens* should be aware of the potential health hazards involved, and should be trained to adopt standard precautions, particularly when dealing with broken or leaking containers.
- Primary specimen containers should be opened in a biological safety cabinet.
- Disinfectants should be available.

Use of centrifuges

- Satisfactory mechanical performance is a prerequisite of microbiological safety in the use of laboratory centrifuges. Centrifuges should be operated according to the manufacturer's instructions.
- Centrifuges should be placed at such a level that workers can see into the bowl to place buckets correctly. Tubes and specimen containers should always be securely capped (screw-capped if possible) for centrifugation.
- Buckets and trunnions should be paired by weight and, with tubes in place, correctly balanced.
- The amount of space that should be left between the level of the fluid and the rim of the centrifuge tube should be given in manufacturer's instructions to be followed.
- Distilled water or alcohol 70% should be used for balancing empty buckets. Saline or hypochlorite solutions should not be used as they corrode metals.
- The interior of the centrifuge bowl should be inspected daily for staining or soiling at the level of the rotor. If staining or soiling is evident then the centrifugation protocols should be re-evaluated.
- Centrifuge rotors and buckets should be inspected daily for signs of corrosion and for hair- line cracks.
- Buckets, rotors and centrifuge bowls should be decontaminated after each use.
- After use, buckets should be stored in an inverted position to drain the balancing fluid.
- Infectious airborne particles may be ejected when centrifuges are used. These particles travel at speeds too high to be retained by the cabinet airflow if the centrifuge is placed in a traditional open- fronted Class I or Class II biological safety cabinet. However, good centrifuge technique and securely capped tubes offer adequate protection against infectious aerosols and dispersed particles.

Glass and "sharps"

- Plastics should replace glass wherever possible. Only laboratory grade (borosilicate) glass should be used, and any article that is chipped or cracked should be discarded.

- Hypodermic needles must not be used as pipettes
- Before discarding disposable syringes/needles, mutilate/ disfigure to avoid reuse or picking up by rag pickers for recirculation.

Films and smears for microscopy

- Fixing and staining of blood, sputum and faecal samples for microscopy do not necessarily kill all organisms or viruses on the smears. These items should be handled with forceps, stored appropriately, and decontaminated and/or autoclaved before disposal.

Chapter 8

BIOMEDICAL WASTE MANAGEMENT

Bio-medical waste is defined as waste that is generated during the diagnosis, treatment or immunization of human beings and are contaminated with patients' body fluids (such as syringes, needles, ampoules, organs and body parts, placenta, dressings, disposables plastics and microbiological waste).

Laboratory waste is a potential reservoir of pathogenic microorganisms and requires appropriate handling. It is the duty of everyone in an institute generating Bio-Medical Waste to take all the steps to ensure that such waste is handled without any adverse effect to human health and environment. All pathology, microbiology laboratories and blood banks are governed by the regulations of biomedical waste (BMW) management rules, 1998.

Safe disposal of laboratory waste

Laboratory wastes are potential hazards. Infectious waste can transmit numerous diseases in the community. Also, those who handle waste are at risk. The increasing use of disposables in health care is also posing an additional burden on the waste management facility. Recycling of these items should be prevented. A small percentage of the waste generated in health care settings is infectious while some of it is non-infectious but hazardous. The most practical approach to the management of biomedical waste is to identify and segregate infectious waste at the point of generation prior to its storage, transportation & disposal. This will drastically reduce the cost of the disposal methods in health care settings.

Principles / concept of waste management

- Segregation of wastes into the prescribed categories must be done at the source i.e. at the point of generation.
- Hospital/ laboratory waste requires management at every step from generation, segregation, collection, transportation, storage, and treatment to final disposal.
- Colour coded bags as per national norms need to be placed in appropriate containers with the appropriate label / logo e.g. biohazard symbol for infectious waste.
- Puncture proof containers made of plastic or metal with a biohazard symbol, in blood collection areas, should be made available for collecting sharp metallic waste.
- Collection systems for the transport of segregated wastes need to be provided for transportation of waste to the site of incinerator.
- A storage area for wastes which already has been disinfected prior to incineration needs to be demarcated.

Table 8.1 – Ten categories of biomedical waste (schedule I of BMW rules 1998)

Option	Waste Category	Treatment and Disposal
Category 1	Human Anatomical wastes (Human tissues, organs, body parts)	Incineration @ / deep burial *
Category 2	Animal waste (animal tissues, organs, body parts carcasses, bleeding parts, fluid , blood and experimental animals used in research, waste generated by veterinary hospitals, colleges, discharges from hospital , animal houses)	Incineration @ / deep burial *
Category 3	Microbiology & Biotechnology Waste (Wastes from lab. Cultures, stocks of specimens of micro-organisms live or attenuated vaccines, human and animal incineration* cell culture used in research and infectious agents from research and industrial laboratories, wastes from production of biologicals, toxins, dishes and devices used for transfer of cultures)	Autoclaving/ microwaving/ incineration @
Category 4	Waste Sharps (Needles, syringes, scalpels, blades, glass etc. that may cause puncture and cuts. This includes both used and unused sharps)	Disinfection (chemical treatment \$\$ / autoclaving/ microwaving and mutilation/ shredding ##)
Category 5	Discarded Medicines & Cytotoxic drugs (Wastes comprising of outdated contaminated and discarded medicines)	Incineration @ / destruction and drugs disposal in secured landfills
Category 6	Soiled Waste (Items contaminated with blood, and body fluids including cotton, dressing, soiled plaster casts, lines, beddings, other material contaminated with blood)	Autoclaving/ microwaving/ incineration @
Category 7	Solid Waste (Waste generated from disposable items other than sharps such as tubings catheters, intravenous sets, etc.)	Disinfection (chemical treatment \$\$ / autoclaving/microwaving and mutilation/ shredding ##)
Category 8	Liquid Waste (Waste generated from laboratory and washing, cleaning house-keeping and disinfecting activities)	Disinfection by chemical treatment \$\$ and discharge to drains.
Category 9	Incineration Ash (Ash from incineration of any bio-medical waste)	Disposal in municipal landfill
Category 10	Chemical Waste (Chemical used in production of biologicals, chemicals used in insecticides etc.)	Chemical treatment \$\$ and discharge into drains for liquids and secured landfill for solids

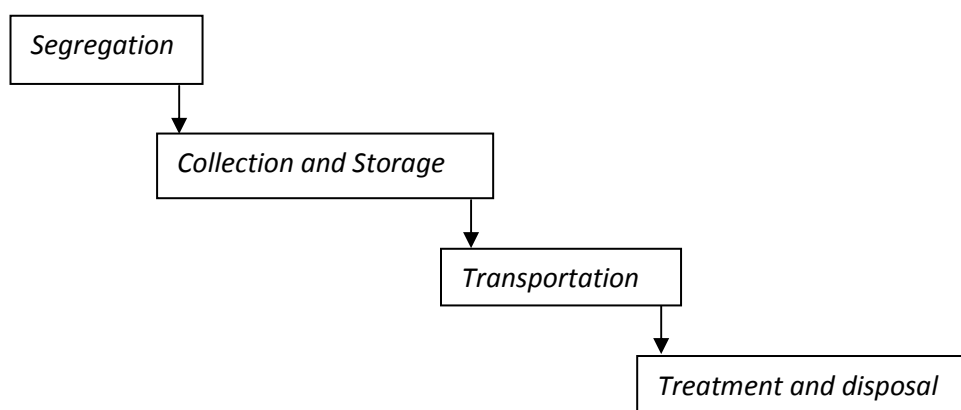
\$\$ Chemical treatment using at least 1% hypochlorite solution or any other equivalent chemical reagent. It must be ensured that chemical treatment ensures disinfection.

Mutilation / shredding must be such as to prevent unauthorized reuse.

@ There will be no chemical pretreatment before incineration. Chlorinated plastics shall not be incinerated.

* Deep burial shall be an option available only in towns with population less than five lakhs and in rural areas

Steps for waste management



Waste segregation

Biomedical waste should be segregated at the point of generation. It consists of placing different types of waste in different containers or colour-coded-bags. This helps in reducing the bulk of infectious waste and contains spread of infection to general waste. This practice reduces the total treatment cost, the impact of waste in the community and the risk of infecting workers.

Table 8.2 – Collection / disposal of biomedical waste

Colour	Type of Container	Waste Category	Treatment options
Yellow	Plastic Bag	Categories 1, 2, 3 & 6	Incineration deep burial
Red	Plastic Bag	Categories 3, 6, 7	Autoclaving/Micro-waving Chemical Treatment
Blue/White Translucent	Plastic Bag/ puncture proof containers	Categories 4, 7	Autoclaving/Micro-waving/ Chemical Treatment & Destruction / shredding
Black	Plastic Bag	Categories 5, 9, 10	Disposal in secured landfill

Collection bags

- Waste collection bags for waste types needing incineration shall not be made of chlorinated plastics.
- Categories 8 and 10 (liquid) do not require containers/bags.
- Category 3, if disinfected locally need not be put in containers/bags
- Solid wastes are collected in leak-resistant heavy duty bags.
- Bags should have labels mentioning date and details of waste.
- The bags to be tied tightly after they are three- fourth full.

Storage and transport

- All containers used for storage of biomedical waste should be provided with a properly covered lid. Such containers should be inaccessible to scavengers and protected against insects, birds, animals and rain.
- If a container is transported from the premises where Biomedical Waste is generated to any waste treatment facility outside the premises, the container should have a label and the information regarding waste category, sender's

name and address. Also the name & address of the person to be contacted in case of emergency should be mentioned on the container.

- Label should be non washable and prominently visible.
- There should not be any spillage during handling and transit of biomedical waste.
- Untreated Biomedical waste should be transported in vehicles authorized for this purpose by the competent authority as specified by the government.
- Untreated Biomedical Waste should not be kept stored beyond a period of 48 hours. If for any reason it becomes necessary to store the waste beyond such period, the authorized person must take permission of the prescribed authority and take measures to ensure that the waste does not adversely affect human health and environment.

Treatment & disposal

- All biomedical wastes should be treated and disposed of strictly in accordance with the guidelines issued by Ministry of Environment and Forest (Notification dated 20 July 1998).
- Waste which cannot be incinerated (plastics), should be pre-treated by disinfection and disposed of in an environmentally sound manner.
- Waste should not be dumped, discharged or disposed in any place other than a site identified for the purpose.
- All treatment and disposal facilities should be located at a specified area away from the general service area of the hospital, public places and residential areas.
- When the treatment option is burial, the pits should be located at sites away from agricultural land, residential areas, ground-and safe water sources. There should be no leakage from the pits into surrounding areas.
- All plastics should be disinfected, shredded and disposed of in an environmentally friendly manner.
- Recycling of disposables e.g. syringes, needles, gloves, etc. should be prevented.
- All liquid waste should be disinfected and flushed in the sinks at the point of generation.
- Biomedical waste should not be disposed off on open land and municipal dustbins.
- Untreated liquid waste should not be let off into sewers.
- All precautions and personal safety measures should be taken (including provision of protective clothing, masks, gloves, gumboots, goggles, etc).

Maintenance of Records

- Every authorized person should maintain records related to the generation, collection, reception, storage transportation, treatment, disposal and or any form of handling of biomedical waste in accordance with the rules.
- All records shall be subject to inspection and verification by the prescribed authority at any time.

Reporting of accidents

In the event of an accident occurring at any location or site where biomedical waste is handled or during transportation, the appropriate authorities must be informed and needful action taken.

Chapter 9

QUALITY ASSURANCE

Quality assurance programmes are an efficient way of maintaining the standards of performance of diagnostic laboratories throughout the world, and of upgrading these standards where necessary. The quality of a microbiological laboratory test not just includes reliability (accuracy) and its reproducibility (precision) but also the speed (test should be rapid enough to be used by the doctor for the treatment), the cost (cost of the test reasonable in relation to the benefit to the patient) and the clinical relevance of the test (test must help in prevention of treatment of the disease).

Factors that affect reliability and reproducibility of the laboratory results

- *Personnel:* The performance of laboratory worker or technician is directly related to quality of education and training received, the person's experience and the conditions of employment.
- *Environmental factors:* Inadequate working space, lighting or ventilation, extreme temperatures, excessive noise levels or unsafe working conditions may affect results
- *Specimens:* The method and time of sampling and the source of the specimen are often outside the direct control of the laboratory, but have a direct bearing on the ability of the laboratory to achieve reliable results. Other factors that the laboratory can control and that affect quality are transport, identification, storage and processing of specimens. The laboratory therefore has a role in educating those taking and transporting specimens. Written instructions should be made available and regularly reviewed.
- *Laboratory materials:* The quality of reagents, chemicals, glassware, stains and culture media all influence the reliability of test results.
- *Equipment:* Lack of equipment or the use of substandard or poorly maintained instruments will give unreadable results.
- *Examination and reading:* Hurried reading of results, or failure to examine a sufficient number of microscope fields can cause errors.
- *Reporting:* Transcription errors or incomplete reports, cause problems.

Quality Assurance in microbiology

Quality assurance is the sum of all those activities in which the laboratory is engaged to ensure that test results are of good quality. There are two types of quality assurance:

- *Internal:* this is called Quality Control. This means that each laboratory has a programme to check the quality of its own tests. Internal quality control is absolutely necessary for good operating procedure and involves, ideally:
 - Continuous monitoring of test quality

- Comprehensive checking of all steps from collection of specimen (wherever possible) to issue of report.
- *External*: this is called Quality Assessment. This means that the laboratory performance is controlled by an external agency. External quality assessment involves:
 - Periodic monitoring of test quality
 - Spot checking of identification tests, & sometimes of isolation techniques.

INTERNAL QUALITY CONTROL

Laboratory operations manual

Each laboratory should have an operations manual, covering the following subjects:

- Biosafety manual which must include instructions for cleaning the workplace, personal hygiene, safety precautions, handling and disposal of infected material.
- Care of equipment
- Guidelines for collection, transport, storage, registration (including rejection criteria), processing, analysis and reporting of specimens.
- Monitoring laboratory equipment and reagents

Care of equipment

A preventive maintenance program to ensure proper functioning of all electrical and mechanical equipment should be established in all microbiology laboratories. Equipment should be checked at prescribed time intervals; certain working parts should be replaced after a specified period of use. Assignments should be made among laboratory personnel to ensure that all inspections are carried out and all data are recorded accurately onto charts or in maintenance manuals. It is important to detect upward or downward trends immediately, so appropriate corrective action can be taken before serious errors result.

Table 9.1 – Quality control procedures of commonly used microbiology equipment for optimal functioning

Equipment	Procedure	Schedule	Tolerance Limits	Technical maintenance
Refrigerators Freezers	Recording of temperature*	Daily or continuous	2 to 8°C -8 to -20°C -60 to -75°C	Every 6 months
Incubators	Recording of temperature*	Daily or continuous	36 ±1°C	Every 6 months
Water baths	Recording of temperature*	Daily or twice daily	36 – 38°C 55 – 57°C	Every 6 months
Autoclaves	<ul style="list-style-type: none"> ▫ Check & adjust water level ▫ Record time & temperature or pressure ▫ Test with spore strip 	<ul style="list-style-type: none"> ▫ Before each run ▫ For each run ▫ At least weekly 	No growth of spores in subculture	Every 6 months
Hot air oven	Record time and temperature*	For each run	± 1°C of setting	Every 6 months

*Format for recording of equipment operating temperature appended in the annexure

Culture Media

Culture media may be prepared in the laboratory from dehydrated powders available commercially or may be purchased ready for use. For best results, careful attention is required to the points given below:

Selection of media

A good agar base can be used as an all purpose medium, for preparing blood agar, chocolate agar and other selective media. One highly selective medium (deoxycholate citrate agar (DCA) or Thiosulfate citrate bile salts (TCBS) agar and one less selective medium are generally all that is required for isolation of Enterobacteriaceae or *Vibrio cholerae* from stool specimen.

Ordering and storage of dehydrated media

- Order quantities that will be used in 6 months or at the most 1 year.
- Overall quantity should be packed up in containers that will be used up in 1-2 months.
- On receipt tighten cap of all containers securely. Dehydrated media absorb water from the atmosphere. In a humid climate, seal the tops of containers of dehydrated media with parafilm/foil etc.
- Write date of receipt of each container.
- Store in a dark, cool, well ventilated place.
- Rotate the stock so that older materials are used first.
- When a container is opened, write the date of opening on it.
- Discard all dehydrated media that are either caked or hardened.
- Keep written records of media in stock.

Preparation of media

- Follow strictly the manufacturers instructions
- Prepare a quantity that will be used up before the shelf life expires

Storage of prepared media

- Protect against sunlight
- Protect against heat. Media containing blood, other organic additives or antibiotics should be stored in the refrigerator.
- The shelf life of prepared media, when stored in a cool, dark place, will depend on the type of container used. Typical shelf lives are:
 - Tubes with cotton plugs - 3 weeks
 - Tubes with loose caps - 2 weeks
 - Petri dishes, if sealed in plastic bags - 4 weeks

Quality control of prepared media

Sterility testing: Carry out routine sterility tests on media to which blood or other components have been added after autoclaving. Take 3-5% of each batch and incubate at 37°C for 2 days. Refrigerate the rest. If more than 2 colonies per plate seen discard the whole batch. Prepared media should also be observed for other signs of deterioration, such as discoloration, turbidity, colour changes, evidence of freeze/thawing and status of dehydration.

Performance testing: The laboratory should keep a set of stock strains for monitoring the performance of media. These strains can be obtained through routine work or from commercial official sources. Each new batch of media should be checked for reactivity and for appropriate support of microbial growth.

Table 9.2 – Quality control of commonly used media

Medium	Control Organisms	Incubation	Expected Reactions
Blood agar	<i>Staphylococcus aureus</i>	24 hrs, CO ₂	Growth and beta haemolysis
TCBS	<i>Vibrio</i> sp (non agglutinable)	24 hrs	Yellow colonies
MacConkey agar	<i>E coli</i> <i>P mirabilis</i>	24 hrs	Red colonies Pale colonies (no swarming)
Deoxycholate citrate agar	<i>E coli</i> <i>S typhimurium</i> / <i>Shigella flexneri</i>	24 hrs	No growth Colourless colonies
Mueller-Hinton agar	<i>E coli</i> ATCC 25922 <i>S. aureus</i> ATCC 25923 <i>P aeruginosa</i> ATCC 27853	24 hrs	Acceptable zone sizes

Stains and reagents

Testing should be carried out each time a new batch of working solution is prepared, and control smears should be stained. Control smear for Gram stain should include a smear with a mixture of Gram positive cocci & Gram negative bacilli. Control smear for Gram stain can be prepared from mixed culture of staphylococci and *Escherichia coli*.

Stains and reagents should be discarded when:

- The manufacturer's expiry date is reached.
- Visible signs of deterioration appear (turbidity, precipitate, discoloration).

Diagnostic antigens and antisera

In order to obtain the best results from antigens and antisera, always follow the manufacturer's instructions.

- Store at the recommended temperature. Some serological reagents do not tolerate freezing. Avoid repeated freezing and thawing. Before freezing, divide antiserum into aliquots sufficient for a few tests.
- Discard when the manufacturer's expiry date is reached.
- To test agglutinating antisera, always use pure, fresh known cultures.
- Always include a serum control of known reactivity in each batch of tests. The serum may be from patient or from a commercial source.
- Paired sera from the same patient, taken during acute and convalescent phases of the disease, should be tested with the same batch of reagents.
- Each batch of serological test should include:
 - A negative serum (specificity control)
 - A weakly reactive serum (sensitivity control)
 - A strongly reactive serum (titration control), which should read within one dilution of its titre when last tested
- Always record all control serum titres

Antimicrobial susceptibility testing

The final result of a disc diffusion test is influenced by a large number of variables. Some of the factors, such as the inoculum density and the incubation temperature, are easy to control, but a laboratory rarely knows the exact composition of a commercial medium or the batch-to-batch variations in its quality, and it cannot take for granted the antimicrobial content of the discs. The results of the test must therefore be monitored constantly by a quality control programme, which should be considered part of the procedure itself.

The precision and accuracy of the test are controlled by the parallel use of a set of control strains, with known susceptibility to the antimicrobial agents. These quality control strains are tested using exactly the same procedure as for the test organisms. The zone sizes shown by the control organisms should fall within the range of diameters given in Table 9.3. When results regularly fall outside this range, they should be regarded as evidence that a technical error has been introduced into the test, or that the reagents are at fault. Each reagent and each step in the test should then be investigated until the cause of the error has been found and eliminated.

The routine use of modified Kirby Bauer method is recommended. To avoid errors, the following guidelines should be used:

- Discs should be of correct diameter and correct potency
- The stock supply should be stored frozen (-20°C)
- The working supply should be kept no longer than one month in a refrigerator (2-8°C)
- Only Mueller Hinton agar whose performance has been tested should be used.
- The inoculum should be standardized against the prescribed turbidity standard
- Zone sizes should be measured exactly and should be compared to a table of critical diameters. Zone diameters for each of the test organism should fall within the control limits.
- Only pure cultures of rapidly growing bacteria give reliable readings.
- The three standard control strains are :
 - *E. coli* ATCC 25922, NCTC 6571
 - *S. aureus* ATCC 25923, NCTC 10418
 - *P. aeruginosa* ATCC 27853, NCTC 10622
- Tests should be carried out with the three standard strains when:
 - A new batch of discs is put to use
 - A new batch of medium is put to use
 - Once a week, in parallel with the routine antibiograms

Reading the plates

After 16 to 18 hours incubation, the diameters of the inhibition zones should be measured with a ruler and recorded, together with the date of the test, on a special quality control chart. This chart should display data for each disc-strain combination. The chart is labelled in millimetres, with an indication of the range of acceptable zone sizes. When the results consistently fall outside the acceptable limits, action should be taken to improve the quality of the test.

Table 9.3 – Zone inhibition diameter limits for control strains

Antibiotic	Diameter of zone of inhibition (mm)			
	Disc potency	<i>S. aureus</i> (ATCC 25923)	<i>E. coli</i> (ATCC 25922)	<i>P. aeruginosa</i> (ATCC 27853)
Amikacin	30 µg	20-26	19-26	18-26
Ampicillin	10 µg	27-35	16-22	-
Benzylpenicillin	10 IU	26-37	-	-
Cefalothin	30 µg	29-37	17-22	-
Ceftriaxone	30 µg	22-28	29-35	17-23
Cefuroxime	30 µg	27-35	20-26	-
Chloramphenicol	30 µg	19-26	21-27	-
Ciprofloxacin	100 µg	22-30	30-40	25-33
Clindamycin	2 µg	24-30	-	-
Co-trimoxazole	25 µg	24-32	24-32	-
Erythromycin	15 µg	22-30	8-14	-
Gentamicin	10 µg	19-27	19-26	16-21
Nalidixic acid	30 µg	-	22-28	-
Nitrofurantoin	300 µg	18-22	20-25	-
Norfloxacin	10 µg	17-28	28-35	22-29
Oxacillin	1 µg	18-24	-	-
Tetracycline	30 µg	19-28	18-25	-

Grossly aberrant results, which cannot be explained by technical errors in the procedure, may indicate contamination or sudden changes in the susceptibility or growth characteristic of the control strain. If this occurs, a fresh stock-strain should be obtained from a reliable source.

Maintenance and use of stock cultures

Stock strains can be obtained from a combination of sources:

- Official culture collections like ATCC etc.
- Reference laboratories
- Properly documented isolates from clinical specimens (select strains so that the maximum number of morphological, metabolic and serological characters can be tested with the minimum number of cultures.

They are commercially available in the form of pellets of desiccated pure cultures. Cultures for day-to-day use should be grown on slants of nutrient agar (tryptic soy agar is convenient) and stored in the refrigerator. They should be subcultured on to fresh slants every 2 weeks.

Preservation

Long term preservation

Long term preservation methods permit intervals of months or even years between subcultures.

The best methods for preservation are lyophilization (freeze drying) or storage at -70°C or below, in an electric freezer or liquid nitrogen. Alternative methods include:

1. Glycerol at -20°C
 - Grow a pure culture on an appropriate solid medium
 - When the culture is fully developed, scrape it off with a loop.

- Suspend small clumps of the culture in sterile neutral glycerol
- Distribute in quantities of 1-2 ml in screw capped tubes or vials.
- Store at -20°C
- Avoid repeated freezing and thawing
- Transfer after 12-18 months
- 2. Mineral oil at room temperature
 - Prepare tubes of heart infusion agar with a short slant
 - Sterilize mineral oil in hot air oven (170°C for 1 hour)
 - Grow a pure culture on the agar slant
 - When good growth is seen, add sterile mineral oil to about 1 cm above the tip of the slant
 - Subculture when needed by scraping growth from under the oil.
 - Store at room temperature.
 - Transfer after 6-12 months
- 3. Stab cultures at room temperature (use for non fastidious organisms like Staphylococcus and Enterobacteriaceae only)
 - Prepare tubes with a deep butt of carbohydrate free agar. Tryptic soy agar (soybean casein digest agar) is recommended.
 - Stab the organism into the agar at several places.
 - Incubate overnight at 37°C
 - Close tube with screw cap.
 - Dip cap with molten paraffin wax to seal.
 - Store at room temperature.
 - Transfer after 1 year.

Short term preservation

Working cultures for daily routine tests (for rapid growing organisms) can be prepared in the following ways:

- Inoculate on tryptic soy agar slants in screw capped tubes
- Incubate overnight at 37°C
- Store in refrigerator
- Transfer every 2 weeks

Use of reference laboratory

Submit the following cultures to a regional or central reference laboratory:

- Specimens for infrequently requested or highly specialised tests (e.g. virology etc)
- Specimens needing further confirmation, specification, grouping, or typing of pathogens of great public health importance e.g. *Salmonella*, *Shigella*, *Vibrio cholerae*, etc.

EXTERNAL QUALITY ASSESSMENT (PROFICIENCY TESTING)

The purposes of a quality assessment programme are:

- To provide assurance to both physicians and the general public that laboratory diagnosis is of good quality.
- To assess and compare the reliability of laboratory performance on a national scale.
- To identify common errors.
- To encourage the use of uniform procedures.
- To encourage the use of standard reagents.
- To take administrative measures (which may include revocation of the operating licence) against substandard laboratories.
- To stimulate the implementation of internal quality control programmes.

Organization

A quality assessment programme consists of a number of surveys in which coded specimens are distributed by mail to participating laboratories. These specimens should be incorporated into the laboratory routine, and handled and tested in exactly the same way as routine clinical specimens.

The surveys should be conducted in accordance with the following recommendations:

- Surveys should be carried out 12 times per year ideally, but at least 4 times per year.
- A minimum of 3 specimens should be included in each survey.
- The reporting period should be short, for example 1 week following receipt of specimen.
- Instructions and report forms should be included with each survey. The report sheet should be in duplicate, with a clearly stated deadline.

Cultures

Cultures could be included for identification and for susceptibility testing against a limited range of antibiotics; they may be pure cultures or mixtures of two or more cultures.

Cultures could represent the following categories:

1. Bacterial species that are of great public health potential, but which are not often seen in routine practice, for example *Salmonella paratyphi* A, *Salmonella typhi*, *Vibrio cholerae*, etc.
2. A mixture of *Shigella*, *E. coli*, and *Klebsiella* may be used to test the skill of a laboratory in isolating pathogenic microorganisms from a number of commensal organisms.
3. A mixture of nonpathogenic organisms may be used to test for ability to recognize negative specimens.

Sera

Serological tests for the following infections could be part of an external quality assessment programme:

- Leptospira
- Dengue
- Typhoid
- Syphilis
- Rubella
- Hepatitis

Rating and reporting of results

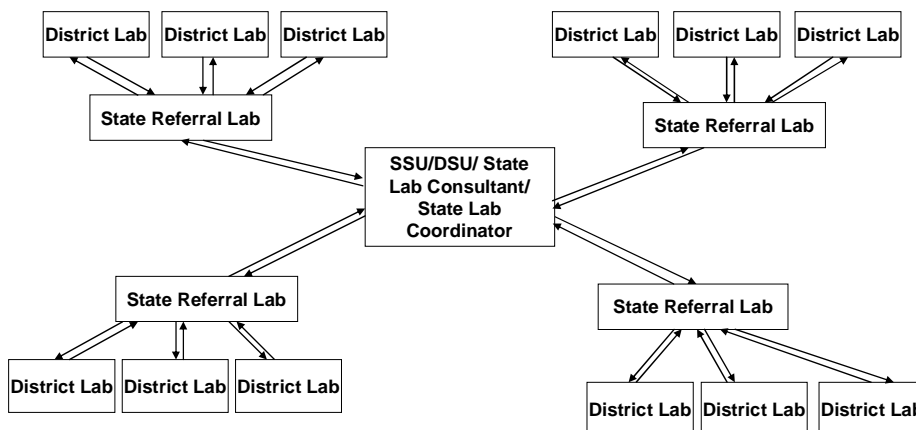
As soon as all reports are received from participating laboratories, the correct answers should be sent to the laboratories. Within one month after that, a final report should be sent to the laboratories with an analysis of the results. A performance score is given to each laboratory. Each laboratory should have a code number known only to itself. Thus it can recognize its own performance in relation to others, but other laboratories remain anonymous.

STATE REFERRAL LAB NETWORK – CONCEPT NOTE

IDSP is aimed to improve the disease surveillance in the country and plans to strengthen the public health laboratories at different levels. However, the implementation experience has shown that the development of public health laboratories is not at the same level at different states.

Most of the labs are performing clinical testing but they lack facilities for diagnosis of epidemic prone diseases. In most states the laboratories have been found to be lacking in manpower and adequate infrastructure and are also not in a position for regularly performing the various tests as planned under the project.

STATE REFERRAL LAB NETWORK – A MODEL (Existing Medical colleges, Number variable)



The current strategy planned is to develop 50 priority district labs in 35 states. To provide access to diagnostic facilities for epidemic prone diseases during outbreak situations to the remaining districts, state based referral lab network is being piloted in 9 states. States are to identify functional labs existing in the medical colleges and various other centres and link the adjoining districts to them for providing the lab services.

Strategy for identification of referral laboratories and networking of districts

Approach

- To identify the existing functional labs in the state as the state referral labs.
- Number of referral labs would be flexible based on the strengths of states.
- Link the districts to the referral labs on an output based arrangement.

Selection Criteria for the State Referral Labs

- Willingness to participate.
- Being a well-functioning microbiology laboratory in terms of adequate infrastructure and human resources and should be regularly performing bacterial culture & sensitivity tests.
- Designate a focal point, a faculty from department of microbiology, who would be responsible for IDSP related activities and will liaison with state lab coordinator
- Agree to comply with the State Biomedical Waste Management Guidelines
- Agree to participate in the External Quality Assessment Scheme
- Agree to sign an MoU with State Surveillance Unit
- Already performing or be able to perform at least the given tests within 3 months of inspection:
 - Cholera and other enteropathogens: culture, sensitivity & serotyping
 - Typhoid: serological tests (TyphiDot), culture & antimicrobial sensitivity (including isolate confirmation with specific antisera)
 - Bacterial meningitis: rapid latex agglutination test, CSF examination - wet mount, gram stain, culture & antimicrobial sensitivity
 - Hepatitis A: IgM μ capture ELISA
 - Hepatitis E: IgM μ capture ELISA
 - Measles: IgM μ capture ELISA
 - Dengue: IgM μ capture ELISA
 - Diphtheria: Smear examination and culture, toxigenicity testing
 - Leptospirosis: ELISA
 - Any other tests for locally prevalent epidemic prone disease (to be identified by the state)

Commitment of Identified Referral Laboratory

- Services to be provided to all the identified attached districts:
 - Sample testing referred by districts for confirmation of outbreak investigations (not for clinical cases)
 - Receipt of outbreak related samples including mechanism of receiving samples on a holiday or after duty hours
 - Provide logistic support for transport of microbiological samples (e.g. provide stool transport media etc)
 - Mentoring of microbiologist / lab technicians towards adequate sample collection and transport
 - Strengthen internal quality control following SOPs
- Co-ordinate with the State Laboratory Coordinator under IDSP
- Report routine laboratory surveillance data weekly on IDSP format ('L' form)
- Report the lab results of outbreak related samples expeditiously to SSO / DSO, maintaining confidentiality
- Report the details of outbreak samples tested from the linked districts on a quarterly basis on prescribed format (attached) to SSU and CSU
- Ensure performance standards:
 - For lab to be functional at all times for public health disease surveillance, maintain adequate

- infrastructure,
 - equipment and
 - kits and consumables
- Participation in EQAS
- Application of the State Biomedical Waste Management Rules

Role of State Surveillance Unit (SSU)

- Designate state level lab coordinator from state services (preferably a senior microbiologist) who will coordinate the referral lab network activities.
- Establish a formal coordination mechanism between public health and medical education system
- Develop a draft plan for referral lab network by identifying the existing functional labs as referral labs and linking the adjoining districts to them.
- Organize sensitization meeting of all stake holders
- Finalize state specific plan, get approved from state authorities and send to CSU
- A team of three experts to carry out initial assessments of compliance of performance level criteria and certify the lab.
 - State Surveillance Officer (SSO)
 - State lab coordinator and
 - State microbiologist recruited under IDSP / senior microbiologist from the state
- Sign an MoU with referral labs
- Provide the referral laboratories the state waste management guidelines
- Provide the referral laboratories the necessary reporting forms (L forms)
- Facilitate the districts in establishing a system of collection and transport of samples from linked districts to the referral labs through video conferencing etc.
- Reimburse the referral laboratory every quarter based on reporting on the number of tests carried out for outbreak investigation in linked districts
- Recruit one state level microbiologist under the project who will help in coordination of referral network at the state level
- Monitor the progress of each referral laboratory; provide an oversight role to ensure timely quality reporting.
- Ensure proper use of funds provided to referral laboratories
- Organise monthly video conferences with focal person from referral labs and the district surveillance officers to enhance the implementation of the referral lab network, including discussions on district level strategies for prompt identification of outbreaks.
- Organize annual state level review workshop for the focal points of the referral laboratories, DSO s and microbiologists and epidemiologists recruited under IDSP

Specific actions of District Surveillance Unit (DSU)

The implementation of referral lab network at the level of the district is the responsibility of the district surveillance officer

1. Establish good coordination with the focal point from the respective referral lab

2. In districts where the priority lab is being strengthened under IDSP, the samples for outbreak investigations are to be referred to the district priority lab
3. Ensure specimen collection (human specimens) and transport during all outbreak investigations in the district
4. Ensure availability of disposables required for specimen collection and transport at district headquarters
5. Ensure follow up with the focal points from the referral labs to obtain the results in a timely manner and incorporate the lab results in the outbreak report sent to SSU and CSU
6. Actively participate in the monthly video conferences organised by the SSU to enhance the implementation of the referral lab network, including reporting on district level strategies for prompt identification of outbreaks

Specific actions of Central Surveillance Unit (CSU)

- Facilitate development of state specific lab plans
- Develop guidelines for referral system for
 - Specimen collection and transport to be used at the districts
 - Expenditure of funds
- Develop prototype SOPs for lab tests.
- Provide quarterly lab reporting formats.
- Identify EQAS organizers for the referral labs
- Monitor the progress

Financial Component

1. Annual grant to each of the identified state referral lab to ensure performance standards. This amount could be utilized for the following:
 - Remuneration of part time staff/overtime
 - Office expenses
 - Minor expenses on instrument repair
 - Procurement of kits and reagents
 - Data collection and communication (phone/internet)
 - Money will be released to the State Surveillance Units as GIA
 - Expenditure will be monitored by respective SSO in coordination with state lab coordinator.

Note

** No procurement of equipment*

** For purchase of reagents and operating costs, use the General Finance Rules (GFR) of Gol*

2. Subsequent reimbursements are based on
 - The tests carried out for epidemic prone disease during outbreaks (not for clinical cases)
 - As determined by DSO, SSO and identified state lab coordinator.
 - Prorata basis, (rates to be decided by State health society)

LIST OF 50 PRIORITY LABORATORIES IN 35 STATES UNDER IDSP

S.No.	Name of States	Name of District Labs	No of district labs identified
1.	Andhra Pradesh	<ul style="list-style-type: none"> ▫ District Public Health Lab, Mahabubnagar ▫ District Public Health Lab, Ongole 	2
2.	Himachal Pradesh	<ul style="list-style-type: none"> ▫ DSU, Mandi ▫ District hospital Lab, Bilaspur 	2
3.	Karnataka	<ul style="list-style-type: none"> ▫ District Public Health Lab, Chiturdurga ▫ District Public Health Lab, Udupi 	2
4.	Madhya Pradesh	JP hospital, Bhopal	1
5.	Maharashtra	<ul style="list-style-type: none"> ▫ District Public Health Lab, Nashik ▫ District Public Health Lab, Beed 	2
6.	Uttarakhand	<ul style="list-style-type: none"> ▫ District Hospital, Pauri ▫ District Hospital, Almora 	2
7.	Tamil Nadu	<ul style="list-style-type: none"> ▫ District Public Health Lab, Cuddalore ▫ District Public Health Lab, Ramanathapuram 	2
8.	Mizoram	<ul style="list-style-type: none"> ▫ District hospital Lab, Lunglei ▫ Civil hospital, Aizwal 	2
9.	Kerala	<ul style="list-style-type: none"> ▫ Regional Public Health Lab, Kozhikode ▫ Regional Public Health Lab, Ernakulam 	2
10.	Chattisgarh	Dept of Microbiology, JL Medical College, Raipur	1
11.	Goa	<ul style="list-style-type: none"> ▫ Goa Medical College, Bambolim ▫ Hospicio hospital, Margao 	2
12.	Gujarat	<ul style="list-style-type: none"> ▫ District Public Health Lab, Mehasana ▫ District Public Health Lab, Sabarkantha 	2
13.	Haryana	<ul style="list-style-type: none"> ▫ District Public Health Lab, Hissar ▫ Haryana Govt PHL, Karnal 	2
14.	Rajasthan	<ul style="list-style-type: none"> ▫ District Public Health Lab, Ajmer ▫ District Public Health Lab, Sikar 	2
15.	West.Bengal	<ul style="list-style-type: none"> ▫ Dist Hospital Pathology Lab, Malda ▫ Dist Hospital Pathology Lab, Berhampore 	2
16.	Manipur	District hospital, Churachandpur	1
17.	Meghalaya	Tura Civil Hospital, Tura	1

18.	Orissa	District Headquarters Hospital, Koraput	1
19.	Tripura	RGM hospital, Kailashahar	1
20.	Chandigarh	Civil hospital, Manimajra, Chandigarh	1
21.	Pondicherry	IGG general hospital, Pondicherry	1
22.	Delhi	Pt. Madan Mohan Malaviya Nagar Hospital, Delhi	1
23.	Uttar Pradesh	<ul style="list-style-type: none"> ▫ Regional lab, Swasthya Bhawan, Lucknow ▫ District Hospital, Ghaziabad 	2
24.	Jammu & Kashmir	Provincial lab, Srinagar	1
25.	Arunachal Pradesh	General hospital, Naharlagun	1
26.	Assam	KK Civil hospital, Golaghat	1
27.	Sikkim	STNM hospital, Gangtok	1
28.	Nagaland	<ul style="list-style-type: none"> ▫ Dist Hospital, Dimapur ▫ State IDSP Lab, Kohima 	2
29.	Dadra & Nagar Haveli	Dist Civil Hospital, Silvassa	1
30.	Punjab	Civil Hospital, Mohali	1
31.	Andaman & Nicobar Islands	Dist Hospital lab, Port Blair	1
32.	Daman & Diu	District hospital Lab, Daman	1
33.	Jharkhand	District Hospital, Ranchi	1
34.	Bihar	Patna Medical College	1
35.	Lakshdweep	Indira Gandhi Hospital, Kavaratti	1
	Total		50

STATE REFERRAL LAB NETWORK UNDER IDSP

(for investigation of outbreaks in the linked districts)

1. Gujarat

<i>S. No.</i>	<i>Name of Institution</i>	<i>Linked districts</i>
1	B.J. Medical College, Ahmedabad	Ahmedabad Rural, Banaskantha, Gandhinagar, Patan, Mahesana, Sabarkantha
2	NLH Medical College Ahmedabad	Ahmedabad Municipal Corporation area, Kheda, Anand
3	Govt. Medical College Vadodara	Narmada, Vadodara, Municipal Corporation, Panchmahal, Dahod, Bharuch
4	Govt. Medical College Surat	Surat Rural, Tapi, Navsari, Valsad, Dangs
5	Govt. Medical College Rajkot	Rajkot Rural, Rajkot corporation, Kutch, Surendranagar
6	Govt. Medical College Bhavnagar	Bhavnagar rural, Bhavnagar Corporation, Amreli, Junagadh, Junagadh corp.
7	Govt. Medical College Jamnagar	Jamnagar Rural, Jamnagar Corporation, Porbander
8	Surat Municipal Corporation Medical college (SMIMER)	Surat municipal corporation area

2. Punjab

<i>S.No.</i>	<i>Name of the Institution</i>	<i>Linked districts</i>
1	Medical College Amritsar	Amritsar, Taran Taaran, Kapurthala, Gurdaspur and Jalandhar
2	Medical College Faridkot	Faridkot, Ferozepur, Bathinda, Muktsar and Moga.
3	Medical College Patiala	Patiala, Sangrur, Mansa, Barnala and F.G Sahib
4	DMC / CMC, Ludhiana	Ropar, Nawan Shahar, Ludhiana and Hoshiarpur

3. Andhra Pradesh

<i>S. No.</i>	<i>Name of the Institution</i>	<i>Linked districts</i>
1	Fever Hospital, Hyderabad	Hyderabad, Nalgonda , Rangareddy
2	Gandhi Medical College, Secunderabad	Nizamabad, Medak
3	Kakatiya Medical College, Warangal	Warangal, Karimnagar, Khammam
4	Kurnool Medical College, Kurnool	Kurnool, Anantapur
5	Sri Venkateswara Medical College, Tirupathi	Chittoor, Nellore, Cuddapah
6	Andhra Medical College, Visakhapatnam	Visakhapatnam, Vizianagaram, East Godavari
7	Guntur Medical College, Guntur	Guntur, Krishna, West Godavari
8	Rajiv Gandhi Institute of Medical Sciences, Srikakulam	Srikakulam
9	Rajiv Gandhi Institute of Medical Sciences, Adilabad	Adilabad

4. Karnataka

<i>S. No.</i>	<i>Name of the Institution</i>	<i>Linked districts</i>
1	Bangalore Medical College	Bangalore Rural , Bangalore Urban ,Kolar, Ramanagar, Chikkaballapura, Tumkur
2	Shimoga Institute of Medical Sciences	Chikmagalur , Shimoga, Davanagere
3	Hassan Med College	Hassan, Kodagu, Dakshina Kannada
4	Mysore Medical College	Mysore, Mandya, Chamarajnagar
5	Vijaynagar Institute of Medical Sciences , Bellary	Bellary, Koppal, Raichur
6	Bidar Med College	Bidar, Gulbarga
7	Belgaum Institute of Medical Sciences	Bijapur, Belgaum, Bagalkote
8	Karnataka institute of Medical Sciences (Govt)	Dharwar, Uttara Kannada , Haveri, Gadag

5. Maharashtra

<i>S. No.</i>	<i>Name of the Institution</i>	<i>Linked districts</i>
1	B.J. Medical College, Pune	Pune, Satara, Ahmednagar, Ratnagiri
2	Vaishampayan Medical College, Solapur	Solapur, Osmanabad, Latur
3	Govt Medical College, Dhule	Dhule, Nandurbar
4	Govt Medical College, Miraj	Sangli, Kolhapur, Sindhudurg
5	Govt Medical College, Aurangabad	Aurangabad, Jalna, Jalgaon
6	Govt Medical College, Nanded	Nanded, Parbhani, Hingoli
7	Govt Medical College, Akola	Akola, Amravati, Buldhana, Washim
8	Govt Medical College, Nagpur	Nagpur, Bhandara, Gondia
9	Sevagram Medical College, Wardha	Wardha, Chandrapur, Yavatmal, Gadchiroli
10	Grant Medical College, Mumbai	Mumbai, Thane, Raigad

6. Rajasthan

<i>S. No.</i>	<i>Name of the Institution</i>	<i>Linked districts</i>
1	J.L.N. Medical College, Ajmer	Bhilwara, Nagaur, Tonk, Ajmer*
2	S.M.S. Medical College, Jaipur	Jaipur, Alwar, Dausa, Jhunjhunu, Bharatpur, Dholpur, Sikar*
3	RNT Medical College, Udaipur	Udaipur, Banswara, Dungarpur, Chittorgarh, Rajsamand, Pratapgarh
4	MBS Medical College, Kota	Bundi, Kota, Baran, Jhalawa, Sawaimadhopur, Karauli
5	Dr. S.N. Medical College, Jodhpur	Jodhpur, Pali, Jalore, Barmer, Jaisalmer, Sirohi
6	S.P.M. Medical College, Bikaner	Bikaner, Churu, Sriganganagar, Hanumangarh

7. Tamil Nadu

<i>S. No.</i>	<i>Name of the Institution</i>	<i>Linked districts</i>
1	Madras Medical College	Thiruvallore, Kancheepuram & Villupuram
2	Vellore Medical College	Vellore & Thiruvanamalai
3	Salem Medical College	Salem, Dharmapuri, Krishnagiri & Namakkal
4	Trichy Medical College	Trichy, Perambalur, Karur & Pudukottai
5	Thanjavur Medical College	Thanjavur, Thiruvarur & Nagapattinam
6	Coimbatore Medical College	Coimbatore, Erode, Nilgiris & Tiruppur
7	Madurai Medical College	Madurai, Dindigul, Theni, Virudhunagar & Sivaganga
8	Tirunelveli Medical College	Tirunelveli, Tuticorin & Kanyakumari

8. Uttarakhand

<i>S. No.</i>	<i>Name of the Institution</i>	<i>Linked districts</i>
1	State Laboratory - IDSP, Pandit Deen Dayal Upadhyay Govt. Hospital, (Coronation Hospital) Dehradun	Dehradun, Tehri-Garhwal, Uttarkashi, Haridwar
2	Govt. Medical College, Srinagar	Chamoli and Rudraprayag
3	Sushila Tiwari Memorial Medical College, Haldwani, District-Nainital	Nainital, Udham Singh Nagar, Bageshwar, Pithoragarh and Champawat

9. West Bengal

<i>S. No.</i>	<i>Name of the Institution</i>	<i>Linked districts</i>
1	North Bengal Medical College	Kochbehar, Jalpaiguri and Darjeeling,
2	Burdwan Medical College	Burdwan and Birbhum
3	Bankura Medical College	Bankura and Purulia
4	Midnapur Medical College	Paschim Midnapur and Purba Midnapur
5	RG Kar Medical College	Nadia and Uttar Dinajpur
6	NRS Medical College	Nadia and Dakshin Dinajpur
7	National Medical College	South 24 Parganas
8	SSKM Medical College	Kolkata
9	Medical College, Kolkata	Howrah and Hoogly

**FORMAT FOR QUARTERLY REPORTING OF OUTBREAK RELATED TESTS
CONDUCTED BY REFERRAL LABS**

Lab name and address							Period of Reporting	
Details of the outbreaks investigated by the referral laboratory								
S.No	<ul style="list-style-type: none"> ▫ Name of disease ▫ District / geographical area ▫ Date of start of outbreak 	Name of specimen collected	Number of specimens tested	Date of Receiving specimens	Transport media used	Test performed	Detailed Test Results*	Date of reporting (to DSO / SSO)
Any other laboratory initiatives / achievements								
Signature of HoD Microbiology								
Name & designation								
Phone / Fax / Email								

* Please mention the name, serotype and antimicrobial susceptibility of the isolates and other relevant epidemiological markers

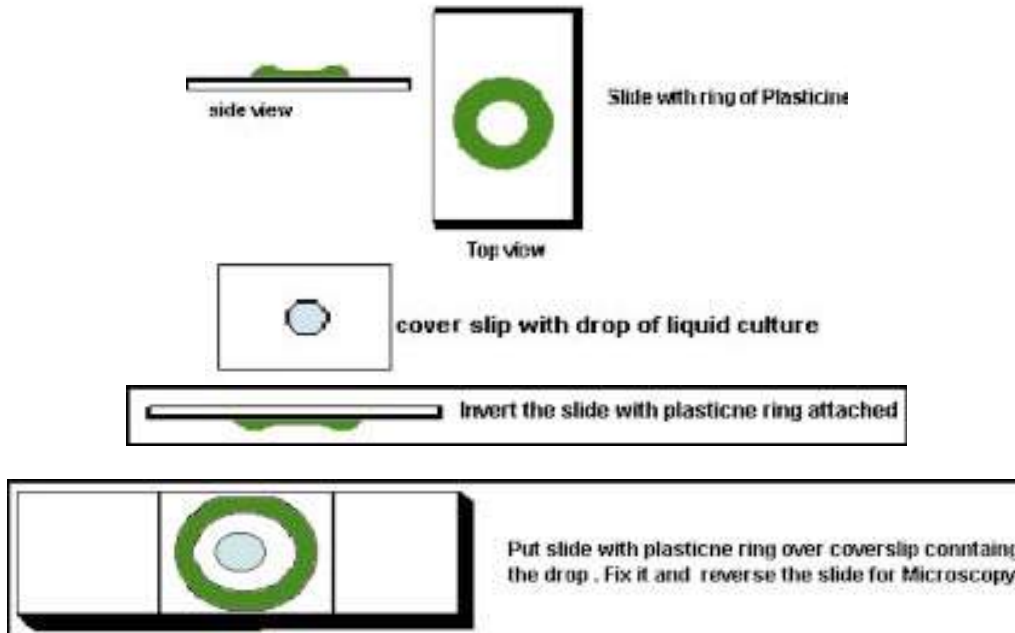
MICROSCOPY

Motility testing - Hanging Drop Preparation

Knowing whether an organism is motile or non-motile can often assist in its identification. The simplest way of examining a bacterial suspension for motile bacteria is as follows:

Procedure

- Take a clean glass slide & make a thin ring of plasticine & apply it over the slide.
- Thickness of the ring should not be more than 1mm, so that there is no difficulty in focusing the slide with 40x high power objective.
- Take a clean cover slip & put on small drop of liquid culture over the cover slip with the help of a small sized inoculating loop (about 1mm diameter).
- Put the slide containing plasticine ring over the cover slip containing the drop of liquid culture without touching the drop and then invert the slide so that the drop hangs.
- Put the condenser low and focus the slide in low power (10x objectives) and try to focus the edge of the drop. Make sure the iris diaphragm of the condenser is sufficiently closed to give good contrast otherwise the organism will not be seen.
- Examine next in high power i.e. 40x objective for checking the motility against the stationary background.



Interpretation

- Motility of *Vibrio cholerae* is typically described as 'darting (shooting star) motility'.
- Finding darting motility is suggestive of *Vibrio cholerae* subject to confirmation of culture.
- Not finding darting motility does not rule out cholera.

The keys to a good hanging drop slide are

- Use a small drop of bacterial suspension, but do not let it dry out, and
- Use a young culture of bacteria.

Note: The movements of small motile bacteria must be distinguished from the on the spot vibratory movement (Brownian movements) which is shown by all microorganism and particles when suspended in a fluid. True bacterial motility is the ability of an organism to move itself in different directions or a single direction.

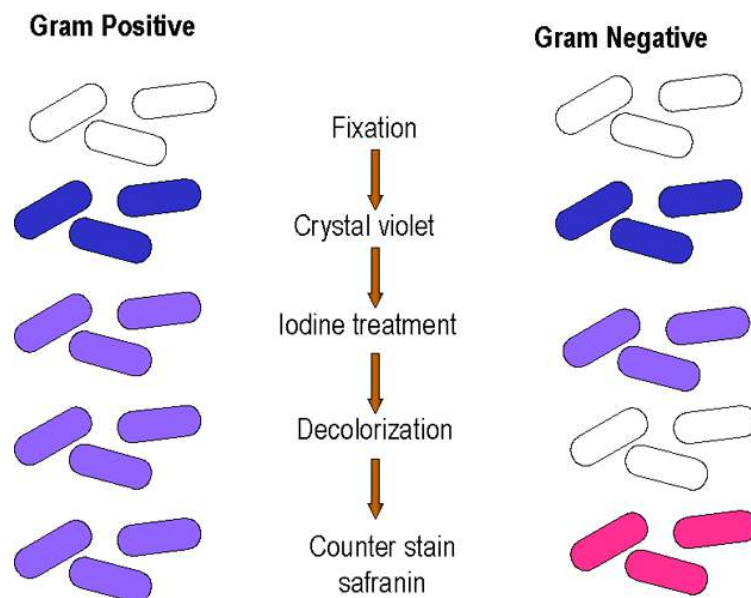
Gram Staining

This is one of the most common staining procedures used for examining specimens suspected to contain bacteriologic agents. Direct microscopic examination of specimens and cultures can provide a rapid presumptive diagnosis. Gram staining can give information regarding the shape of cell, the type of cell arrangement (single chained, clustered) and gram reaction that can provide a quick assessment of what the etiologic agent may be.

Principle

Certain bacteria when treated with one of the basic para rosaniline dyes such as Methyl Violet, Crystal Violet or Gentian Violet (which is a mixture of two preceding dyes) and then with Iodine, fix the stain so that subsequent treatment with a decolourizing agent e.g. alcohol or acetone does not remove the colour. Other organisms however, are decolourized by this process. Thus, if a mixture of various organisms are stained and subjected to decolourizing agents, it is found that some retain the dye, and these are termed Gram-Positive whereas others are completely decolourized and are termed as Gram-Negative.

In order to render the decolourized organism visible, and to distinguish them from those retaining the colour, a counter stain is then applied. This counter stain is usually red so that the gram-negative organism which appears reddish pink in colour may easily be differentiated from gram-positive organism, which retains the original violet stain.



Gram stain - basic steps

Specimens

Smear prepared from any of the following e.g. throat swab, nasal swab, ear discharge, pleural fluid, CSF, urethral discharge, sputum, centrifuged deposit of urine, bacterial culture, vaginal discharge etc. can be stained by this method.

Materials

- Crystal violet (0.5%)
 - Crystal violet – 0.5 gm
 - Distilled water – 100 ml
- Gram's iodine (1%)
 - Iodine crystals – 1gm
 - Potassium iodide – 2 gm
 - Distilled water – 100 ml
- Acetone (100%) or Ethanol (95%)
- Safranine (0.5%)
 - Safranine-0.5gm
 - Ethyl Alcohol – 10 ml
 - Distilled water-make up volume to 100 ml
- Distilled water

Procedure

- Appropriate smear is made on a clean glass slide.
- The smear is fixed by passing the slide over flame 2-3 times quickly.
- Cover the slide with crystal violet solution and allow to act for about 30 seconds.
- Pour off stain and holding the slide at an angle downwards pour on the iodine solution on the slide so that it washes away the crystal violet. Cover the slide with fresh iodine solution and allow to act for 1 minute.
- Wash off the iodine with ethanol and treat with fresh alcohol, tilt the slide from side to side until colour ceases to come out of the preparation. This is easily seen by holding the slide against a white background.
- Alternatively, decolorize with 100% acetone. First tip off the iodine and hold the slide at a steep slope. Then pour acetone over the slide from its upper end, so as to cover its whole surface. De-colorization is very rapid and is usually complete in 2-3 seconds. After this period of contact, wash thoroughly with water under a running tap
- Apply the counter-stain (0.5% safranine) for 30 seconds.
- Wash with water and blot dry.
- Examine the smear under oil immersion microscopy.
- Result
 - Violet stained bacteria - Gram-Positive
 - Reddish pink bacteria - Gram-Negative

Quality Control

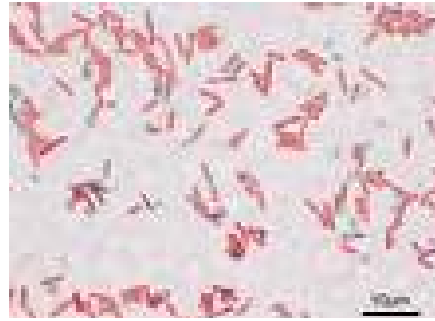
- Use clean, grease-free slides for making smears.
- Prepare smear 2 cm in diameter and not very thick.
- To avoid false positive results, ensure the cleanliness of the microscope especially the oil immersion (100X) objectives which come in contact with smears
- To obviate errors from over decolorizing, a control smear of a known gram positive organism (.e.g. a pure culture of *Staphylococcus aureus*) may be prepared on one end of slide and a smear of gram negative organism e.g. *Escherichia coli* be prepared on the other end of the slide. The smears thus prepared should be stained by the grams

stain as described. The smear prepared from *Staphylococcus aureus* should show violet coloured bacteria whereas the one made from *E. coli* should show pink coloured bacteria.

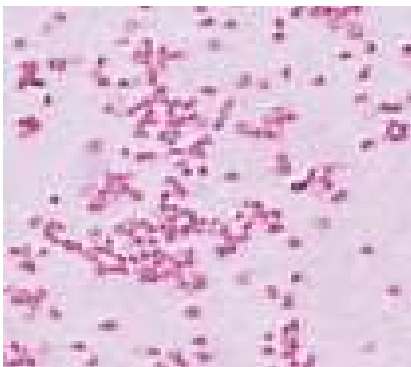
- Check the quality of stains by using appropriate positive and negative control slides at least once a week and whenever a fresh batch of stains is introduced on the work bench



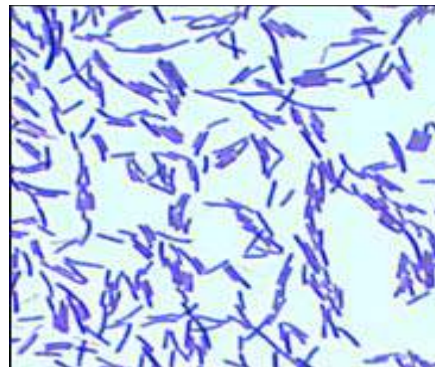
Gram positive cocci (in chains)



Gram negative bacilli



Gram negative cocci (in pairs)



Gram positive bacilli

Preparation of Gram stained smear from CSF specimen

Materials required

Sterile glass slide, dropper, centrifuge, stains, sterile tube

Procedure

1. Sufficient CSF, at least 1-2 ml should be available for centrifugation.
2. Centrifuge in a clean sterile tube at high speed for 3-5 min.
3. Take out the tube carefully; remove the supernatant in a separate sterile tube for antigen detection tests.
4. Take the pellet deposit and use for both culture and smear preparation.
5. Put a drop of the deposit onto the centre of the slide and spread evenly onto a small area. Let it air dry.
6. After the smear has dried, heat fix it.
7. Place the slide over the staining rack. Cover with crystal violet for 1 min.
8. Wash with water and pour iodine solution over it. Keep for 1 min.
9. Decolourize with acetone quickly, again wash with water.
10. Counterstain with safranin for 1 min. Wash with water.
11. Blot dry and observe under oil immersion objective.

Observation

The meningococci appear as gram negative diplococci both inside and outside polymorphs. Heavily encapsulated strains have a distinct pink halo around them. Often the organisms show considerable size variation and may resist decolourization. Report the number of organisms and the number of polymorphs as they have a prognostic value.

Albert's Staining

Principle

The diphtheria bacilli - *Corynebacterium diphtheriae* have well developed granules of volutin (polymetaphosphate) which are seen as round refractile bodies within the bacterial cytoplasm. With basic dyes, they tend to stain more strongly than the rest of the bacterium. With toluidine blue / methylene blue they stain metachromatically a reddish purple colour. This is demonstrated most clearly by special methods such as Albert's or Neisser's. Diphtheria bacillus gives its characteristic volutin reactions best in a young culture (18-24hr) on blood or serum medium.

Albert's staining solution (Albert's I) contains

- Toluidine blue 1.5g
- Malachite green 2.0g
- Glacial acetic acid 10 ml
- Alcohol(95% ethanol) 20 ml
- Distilled water 1000 ml

Dissolve the dyes in alcohol and add to the water & acetic acid. Allow to stand for 1 day and then filter. Alternatively, use commercially prepared stain.

Albert's iodine (Albert's II) contains

- Iodine 6g
- Potassium iodide 9g
- Distilled water 900 ml

Dissolve the KI in I2 and

Procedure

1. Make smear, dry in air and heat fix.
2. Cover with Albert I and allow to act for 3-5 minutes.
3. Wash with water and blot dry.
4. Cover with Albert's iodine for 1 minute. Wash & blot dry.
5. Observe under 100x (oil immersion) objective.
6. The bacilli appear green, granules stain bluish black, and other organisms stain light green. The "barred" appearance is due to the presence of polyphosphate inclusions called metachromatic granules. Note also the characteristic "Chinese-letter" arrangement of cells.



Albert-stained *C. diphtheriae*

COMMON CULTURE MEDIA

If pathogens are to be isolated successfully, standard operating procedures (SOPs) are needed for each culture medium used in the laboratory, its purpose, from where it can be obtained, its preparation and how it is sterilized, dispensed, labelled, stored and performance tested. In the preparation of complex culture media, it is advisable for district laboratories to use ready-made standardized dehydrated media to ensure good performance and reproducibility.

Dehydrated media is hygroscopic, i.e. it absorbs water, when exposed to water it rapidly becomes unfit for use. Adequate precautions must be taken to prevent this, which include:

1. Weighing the medium rapidly and tightly capping the bottle as soon as possible after removing the approximate amount. Do not return small amounts of medium to the stock bottle.
2. Sealing the cap of the container with adhesive tape.
3. Storing media in the coolest driest place available and always out of sunlight, e.g. do not store dehydrated media in the same room as used for steam sterilization, boiling materials, or cleaning glassware etc.

Preparation of culture media

The following are important while preparing culture media:

- Prepare media made from dehydrated products in as damp-free an environment as possible.
- To prevent the risk of inhaling fine particles of dehydrated media, wear a dust mask while handling dehydrated media.
- Wash hands immediately after preparing media.
- Once the ingredients are weighed, do not delay in making up the medium. Follow the manufacturer's instructions exactly.
- Use completely cleaned glassware, plastic or stainless steel equipment that has been rinsed in pure water. The container in which the medium is prepared should have a capacity of at least twice the volume of the medium being prepared.
- Use distilled water.
- Add the powdered ingredients to the water and stir to dissolve. Do not shake but mix by stirring or by rotating the container.
- Autoclave a medium only when the ingredients are completely dissolved. Always autoclave at the correct temperature and for the time specified.
- Dispense medium in bottles or tubes in amounts convenient for use. Know the length of time prepared media can be stored without deteriorating (take into account storage temperature).
- Check the media prepared for sterility and performance using standard strains.

Checking the pH of a culture medium

The pH of most culture media is near neutral. An exception is alkaline peptone water. The simplest way of testing the pH of a culture medium is to use narrow range pH papers or a pH meter. pH should be checked before autoclaving.

Sterilizing culture media

The following methods are used to sterilize culture media:

- *Autoclaving* - It is important to sterilize a medium at the correct temperature and for the correct length of time as instructed in the method of preparation. Under-autoclaving can result in an unsterile medium which will need to be discarded. Over-autoclaving can cause precipitation, alteration of pH, and the destruction of essential components in a medium and will not support the growth of bacteria.
- *Steaming at 100°C* - This is used to sterilize media containing ingredients that would be broken down or inactivated at temperatures over 100°C, e.g. Cary-Blair transport medium. Steaming is also used to re-melt previously bottled sterile agar media. Steaming can be performed in an autoclave with the lid left loose, or in any form of steam sterilizer. The bottles of media with loosened caps are placed on perforated trays above the boiling water. After sterilization once the medium has cooled, the bottle tops are tightened. Steaming times vary according to the type of medium, e.g. 15 minutes for Cary-Blair medium.
- *Filtration* - This provides means of removing bacteria and suspended impurities from fluids. It is used to mainly to sterilize fluid additives that are heat sensitive and cannot be autoclaved. Examples include serum and solutions containing urea and certain carbohydrates. Cellulose filters commonly referred to as membrane filters are the commonly used filters as they filter more rapidly, do not affect the filtrate and absorb very little of the substance being filtered. Membrane filters are available in a variety of pore sizes, with 0.22 µm being required for sterile filtration.

Table A6.1 – Shelf life and pH of common media

Medium	pH	Shelf life (at 2-8°C)*
Cary Blair medium	8.4 ± 0.2	6 months
Alkaline Peptone Water	8.6 – 9.0	2 years
Peptone Water	7.0 – 7.6	2 years
Tryptic Soya Broth	7.1 – 7.5	6 months
Mueller Hinton Agar	7.2 – 7.6	4 weeks
Nutrient Agar	7.2 – 7.6	2 years (NA deeps)
Blood Agar	7.2 – 7.6	4 weeks
MacConkey Agar	7.2 – 7.6	4 weeks
Thiosuphate Citrate Bile Salt (TCBS) agar	8.4 – 8.8	4 weeks
Xylose Lysine Deoxycholate	7.2 – 7.6	4 weeks
Christensen's Urea broth	6.6 – 7.0	6 months
Triple Sugar Iron agar	7.2 – 7.6	3 weeks

* provided there is no change in the appearance of the medium to suggest contamination, alteration of pH or storage temperature

PREPARATION OF SOME COMMONLY USED MEDIA

Cary Blair transport medium

Contents:

- Sodium thioglycollate, disodium hydrogen phosphate, sodium chloride & agar

Preparation:

1. Prepare the medium as instructed by the manufacturer. Dispense the medium in 7 ml amounts in screw cap tubes



Cary Blair medium

2. Sterilise by steaming (with caps loosened) for 15 minutes. When cool, tighten the bottle caps. Label the bottles.
3. Date the medium and record the expiry date. Store in cool dark place with bottle tops screwed tightly to prevent a pH change.

pH: 8.4±0.2 (at 25°C)

Shelf life: 6 months from preparation provided there is no change in colour of the medium to suggest contamination).

Inoculation: Immerse a swab of faecal specimen in the medium, cutting off the swab stick to allow the bottle top to be replaced tightly. Protect the swab from direct light and excessive heat.

Alkaline Peptone Water (APW)

Contents:

- Peptone, Sodium chloride, Distilled water

Preparation:

1. Dissolve the peptone and sodium chloride in the water
2. Adjust the pH of the medium adding sufficient 1mol/litre solution of sodium hydroxide.
3. Dispense the medium in 10 ml amounts in screw capped bottles.
4. Sterilise by autoclaving (with caps loosened) at 121°C for 15 minutes. When cool, tighten the bottle caps. Label the bottles.
5. Date the medium and record the expiry date.
6. Store in cool dark place with bottle tops screwed tightly to prevent a pH change.

pH: range 8.6 -9.0 (at 25°C)

Shelf life: 2 years from preparation, provided there is no change in volume or appearance of the medium to suggest contamination.

Inoculation:

- For use as enrichment media inoculate with 3-4 loopfuls of faecal specimen and incubate at 37°C for 5-8 hours. Subculture several loopfuls (from the surface) on to a plate of TCBS.
- For use as transport media inoculate with about 1 ml of faecal specimen or with a rectal swab. The inoculated medium should reach the laboratory within 10 hours.

Peptone Water

Contents:

- Peptone, Sodium chloride, Distilled water

Preparation:

1. Dissolve the peptone and salt in the water.
2. Dispense in 3 ml amounts in screw-cap bottles(Bijou type are suitable)
3. Sterilize by autoclaving (with caps loosened) at 121° C for 15 minute.
4. When cool, tighten the bottle caps. Date the medium and give it a batch number.
5. Store in a cool dark place.

pH: range 7.0-7.4 (at 25°C)

Shelf-life: Up to 2 years providing the medium shows no change in volume or appearance to suggest contamination.

Mueller-Hinton Agar (MHA)

Contents:

- Beef infusion, casein acid hydrolysate, starch, agar

Preparation:

1. Sterilize by autoclaving at 121°C for 15 minutes.
2. When the medium has cooled to 50-55°C, mix well and dispense aseptically in sterile petri-dishes. Date the medium and give it a batch number.
3. Store the plates at 2-8°C preferably in plastic bags to prevent loss of moisture.

pH: range 7.2-7.6 (at 25°C)

Shelf-life: Up to 4 weeks provided there is no change in the appearance of the medium to suggest contamination or an alteration of pH.

Nutrient Agar

Contents:

- Peptone, beef extract, agar

Preparation:

1. Dispense aseptically in the required amounts
 - 3 ml to make nutrient agar slopes
 - 5 ml to make nutrient agar deeps
 - or the amounts required to make blood agar or other media
2. Date the medium and give it a batch number.
3. Store in a cool dark place.

pH: 7.2-7.6 (at 25°C)

Shelf life (nutrient agar deeps): Up to 2 years, provided there is no change in the appearance of the medium to suggest contamination or deterioration.

Inoculation (of control species in agar deeps):

1. Stab inoculate the organism.
2. Incubate at 37°C until a small colony forms over the stab.
3. Cover with sterile mineral oil.
4. To subculture, tip the oil to one side and pick off a small quantity of growth.

Blood Agar

Contents:

- Nutrient agar / blood agar base, sheep blood

Preparation:

1. Prepare the agar medium as instructed by the manufacturer.
2. Sterilize by autoclaving at 121°C for 15 minutes. Transfer to a 50°C water bath.
3. When the agar has cooled to 50°C, add aseptically the sterile blood (5ml for every 95ml of medium) and mix gently but well. Avoid forming air bubbles.
4. *Important:* The blood must be allowed to warm to room temperature before being added to the molten agar.
5. Dispense aseptically in 15 ml amounts in sterile petri-dishes
6. Date the medium and give it a batch number.
7. Store the plates at 2-8°C, preferably in sealed plastic bags to prevent loss of moisture.

pH: range 7.2-7.6 (at 25°C)

Shelf-life: Up to 4 weeks providing there is no change in the appearance of the medium to suggest contamination, haemolysis, or deterioration.

MacConkey Agar

Contents:

- Bacto peptone, bacto proteoseptone, lactose, bile salt, sodium chloride, neutral red, agar

Preparation:

1. Prepare as instructed by the manufacturer.
2. Sterilize by autoclaving at 121°C for 15 minutes.
3. When the medium has cooled to 50-55°C, mix well and dispense aseptically in sterile petri-dishes. Date the medium and give it a batch number.
4. Store the plates at 2-8°C preferably in plastic bags to prevent loss of moisture.

pH: range 7.2-7.6 (at 25°C)

Shelf-life: Up to 4 weeks provided there is no change in the appearance of the medium to suggest contamination or an alteration of pH.

Some lactose fermenting (LF) organisms	Some non-lactose fermenting (NLF) organisms
<ul style="list-style-type: none">▫ <i>Escherichia coli</i>▫ <i>Klebsiella pneumoniae</i>	<ul style="list-style-type: none">▫ <i>Salmonella species</i>▫ <i>Shigella species</i>▫ <i>Proteus species</i>▫ <i>P. aeruginosa</i>

Tryptic Soya Broth (Blood culture broth)

Contents:

- Tryptic soya broth, sodium polyanetholesulfonate (SPS)

Preparation:

1. Follow the instructions of the manufacturer on the label of each bottle of dehydrated trypticase soy broth.
2. Add 0.25g SPS per litre of medium. SPS is especially important for recovery of *H. influenzae* since it prevents the inoculated blood from clotting.
3. Dispense in 20 ml (pediatric blood culture bottle) and 50 ml (adult blood culture bottle) amounts into suitable containers (e.g. tubes or bottles) with screw-caps with rubber diaphragms. The amount of liquid in the containers should comprise not more than two-thirds of the total volume of the container.
4. Sterilize by autoclaving at 121°C for 15 minutes. Cool and store medium at room temperature.

pH: 7.1-7.5 (at 25°C)

Shelf life: Up to 6 months provided there is no change in the appearance of the medium to suggest contamination or an alteration of pH.

Quality control:

Each new batch of freshly prepared or purchased blood culture medium should be tested for supporting the growth of a variety of pathogens including, e.g., *H. influenzae*, *N. meningitidis*, *S. pneumoniae*, and *S. typhi*.

Thiosulphate Citrate Bile Salt (TCBS) agar

Contents:

- Proteose peptone, yeast extract, sodium thiosulphate, sodium citrate, ox-gall, sucrose, sodium chloride, ferric citrate, bromothymol blue, thymol blue, agar



Growth of *Vibrio cholerae* on TCBS

Preparation:

1. Prepare as described by the manufacturer.
2. Heat the medium with great care. It must not be over heated or autoclaved.
3. Dispense aseptically in sterile petri-dishes. Date the medium and give it a batch number.
4. Store the plates at 2-8°C, in sealed plastic bags to prevent loss of moisture.

pH: 8.4-8.8 (at 25°C)

Shelf-life: Up to 4 weeks providing there is no change in the appearance of the medium to suggest contamination or alteration of pH.

Xylose Lysine Decoxycholate (XLD) Agar

Contents:

- Yeast, l-lysine HCl, lactose, sucrose, sodium deoxycholate, sodium chloride, sodium thiosulphate, ferric ammonium citrate, phenol red, agar

Preparation:

1. Prepare as instructed by the manufacturer. Heat the medium with care and do not over heat or autoclave.
2. As soon as the medium has cooled to about 55°C, mix well, and dispense aseptically in sterile petri dishes. Label and plates 'XLD'.
3. Date the medium and give it a batch number. Store the plates at 2-8°C, preferably sealed in plastic bags to prevent loss of moisture.

pH: range 7.2-7.6 (at 25°C)

Shelf-life: Up to 4 weeks providing there is no change in the appearance of the medium to suggest contamination, deterioration, or alteration of pH.



Shigella flexneri (pale colonies)
& *E. coli* (pink colonies) on XLD

Christensen's Urea Agar

Contents:

- Urea agar base, sterile urea solution

Preparation:

1. Prepare and sterilize the urea agar base as instructed by the manufacturer
2. Transfer to a 50-55°C water bath
3. When the medium has cooled to 50-55°C add aseptically the sterile urea solution and mix well.
4. Dispense aseptically in 3 ml amounts in sterile Bijou bottles or screw-cap tubes
5. Date the medium
6. Store in a cool dark place or at 4-8°C

pH: range 6.6-7.0 (at 25°C)

Shelf life: Upto 6 months provided there is no change in the volume or appearance of the medium to suggest contamination or alteration of pH.

Triple Sugar Iron (TSI) Agar

Contents:

- Peptone, yeast extract, sodium chloride, lactose, glucose, sucrose, ferric citrate, sodium thiosulphate, phenol red, agar

Preparation:

1. Prepare as instructed by the manufacturer
2. Dissolve the required amount of media powder in appropriate volume of distilled water by heating.
3. When cooled to 50 – 55° C, mix well and dispense in 6 ml amounts in large size tubes (approx. 15 x 150 mm)
4. Sterilize by autoclaving (with caps loosened) at 121°C for 15 minutes.
5. Allow the medium to solidify in a sloped position to give a butt 25-30mm long and a slope 20-25 mm long. Date the medium.
6. Store in a cool dark place at 2-8°C

pH: range 7.2-7.6 (at 25°C)

Shelf life: About 3 weeks provided the screw caps are tightly screwed and there is no change in the appearance of medium to suggest contamination.

Simmon's Citrate Agar

Contents:

Ammonium Dihydrogen Phosphate, Dipotassium Phosphate, Sodium Chloride, Sodium Citrate, Magnesium Sulfate, Bromthymol Blue, Agar

Preparation:

1. Prepare as instructed by the manufacturer.
7. Dissolve the required amount of media powder in appropriate volume of distilled water by heating.
2. When cooled to 50 – 55° C, mix well and dispense 2-3 ml in small tubes
3. Autoclave at 121°C for 15 minutes and allow to set as slopes
4. Date the medium and give it a batch number. Store the tubes at 2-8°C in the dark.

pH: range 6.7-7.1 (at 25°C)

Shelf-life: Up to 4 weeks providing there is no change in the appearance of the medium to suggest contamination, deterioration, or alteration of pH.

Results:

A positive reaction is indicated by growth on the slant with an intense blue color (alkaline reaction). A negative reaction is indicated by inhibition to poor growth without change in colour (medium remains green).

TESTS FOR IDENTIFICATION OF SOME COMMONLY ISOLATED BACTERIA

Catalase Test

This test is used to differentiate those bacteria that produce the enzyme catalase from non-catalase producing bacteria.

Requirements:

- Hydrogen peroxide, 3% H₂O₂
- Glass rod and slides

Method:

1. Place a drop of H₂ O₂ on a clean glass slide
2. Using a sterile glass rod and remove several colonies of the test organism and immerse in drop of H₂O₂
3. Look for immediate bubbling

Results:

- Active bubbling = Positive catalase test
- No bubbles = Negative catalase test

Oxidase test

The oxidase test is used to assist in the identification of Pseudomonas, Neisseria, Vibrio, Brucella, and Pasteurella species, all of which produce the enzyme cytochrome oxidase.

Requirements:

- Oxidase reagent
- Filter paper
- Glass rod & slides

Preparation of Oxidase Reagent:

- Tetramethyl-p-phenylenediamine dihydrochloride 0.1 g
- Distilled water 10 ml

Dissolve the chemical in the water. It is therefore best prepared immediately before use.

Note: Some commercially available oxidase reagents are more stable and can be stored for several days at 2-8°C. They must be used as directed by the manufacturer. Stable oxidase reagent strips are also available.

Method (fresh reagent):

1. Place a piece of filter paper in a clean petri dish and add 2 or 3 drops of freshly prepared oxidase reagent.
2. Using a piece of stick or glass rod (not a Ni-Chrome wire loop), remove a colony of the test organism and smear it on the filter paper.
3. Look for the development of a blue-purple colour within a few seconds.



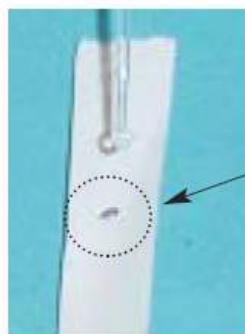
STEP 1: Place filter paper treated with Kovac's oxidase in a Petri dish.



STEP 2: Prepare inoculum and touch filter paper with loop



STEP 3: A positive reaction with Kovac's oxidase is detected within 10 seconds by a color change to purple on the area of the filter paper where growth was rubbed (in step 2).



Results:

- Blue-purple colour = Positive oxidase test (within 10 seconds)
- No blue-purple colour = Negative oxidase test (within 10 seconds)

Citrate utilization test

This test is one of several techniques used occasionally to assist in the identification of enterobacteria. The test is based on the ability of an organism to use citrate as its only source of carbon.

Inoculation:

1. Using a sterile straight wire, first streak the slope with a saline suspension of the test organism and then stab the butt.
2. Incubate at 37°C for 48 hours.

Results:

- Bright blue = Positive citrate test
- No change in colour of medium (remains green) = Negative citrate test

Indole test

Method:

1. Inoculate the test organism in a tube containing 3 ml of sterile peptone water.
2. Incubate at 37°C for up to 48 hours.
3. Test for indole by adding 0.5 ml of Kovac's reagent. Shake gently.
4. Examine for a red colour in the surface layer within 10 minutes.

Results:

- Red ring = positive Indole
- Colourless ring = negative Indole



Positive (red ring)
Negative (colourless ring)

Urease test

Method (using urea agar):

1. Inoculate heavily the test organism in a bijou bottle containing urea agar.
2. Incubate at 37°C for 3-12 hours.
3. Look for a change in colour of the medium.

Results:

- Pink colour = Positive urease test
- No pink colour or change = Negative urease test



Positive (pink)
Negative (no change)

TSI (Triple Sugar Iron) test

Inoculation:

Use straight wire to inoculate TSI medium, first stabbing the butt and then streaking the slope in a zigzag pattern. After inoculation make sure that the tube tops are left loose. Incubate overnight at 37°C.

Results:

Reactions are based on fermentation of lactose, sucrose and glucose and production of hydrogen sulphide (H₂S)

- A yellow butt (acid production) and red – pink slope indicate the fermentation of glucose only. This reaction is seen with *Salmonella* and *Shigella* species and other enteric pathogens
- Cracks and bubbles in the medium indicate gas production from glucose fermentation.
- A yellow slope and a yellow butt indicate the fermentation of glucose and lactose and/or sucrose. This occurs with *E. coli*, *Vibrio cholerae* and other enterobacteria.
- A red pink slope and but indicate no fermentation of any of the sugars. This is seen with most strains of *Pseudomonas aeruginosa*.
- Blackening along the stab line or throughout the medium indicates H₂S production e.g. *Salmonella typhi* produces a small amount of blackening while *Salmonella typhimurium* and *Proteus* (normal flora in stool) causes extensive blackening.



Salmonella typhi

TEMPERATURE RECORD OF EQUIPMENT

DAILY TEMPERATURE CHART FOR REFRIGERATOR / FREEZER / INCUBATOR / WATERBATH #: _____ Thermometer #: _____ Acceptable temp: _____ Month: _____ Year: _____			
Date	Temp Observed	Initials	Comments
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